Pathogenic infection characteristics and risk factors for bovine respiratory disease complex based on the detection of lung pathogens in dead cattle in Northeast China

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ABSTRACT

Bovine respiratory disease complex (BRDC) involves multiple pathogens, shows diverse lung lesions, and is a major concern in calves. Pathogens from 160 lung samples of dead cattle from 81 cattle farms in Northeast China from 2016 to 2021 were collected to characterize the molecular epidemiology and risk factors of BRDC and to assess the major pathogens involved in bovine suppurative or caseous necrotizing pneumonia. The BRDC was diagnosed by autopsy, pathogen isolation, PCR, or reverse transcription-PCR detection, and gene sequencing. More than 18 species of pathogens, including 491 strains of respiratory pathogens, were detected. The positivity rate of bacteria in the 160 lung samples was 31.77%, including Trueperella pyogenes (9.37%), Pasteurella multocida (8.35%), Histophilus somni (4.48%), Mannheimia haemolytica (2.44%), and other bacteria (7.13%). The positivity rate of Mycoplasma spp. was 38.9%, including M. bovis (7.74%), M. dispar (11.61%), M. bovirhinis (7.94%), M. alkalescens (6.11%), M. arginini (0.81%), and undetermined species (4.68%). Six species of viruses were detected with a positivity rate of 29.33%, including bovine herpesvirus-1 (BoHV-1; 13.25%), bovine respiratory syncytial virus (BRSV; 5.50%), bovine viral diarrhea virus (BVDV; 4.89%), bovine parainfluenza virus type-3 (BPIV-3; 4.28%), bovine parainfluenza virus type-5 (1.22%), and bovine coronavirus (2.24%). Mixed infections among bacteria (73.75%), viruses (50%), and M. bovis (23.75%) were the major features of BRDC in these cattle herds. The risk analysis for multi-pathogen co-infection indicated that BoHV-1 and H. somni; BVDV and M. bovis, P. multocida, T. pyogenes, or Mann. haemolytica; BPIV-3 and M. bovis; BRSV and M. bovis, P. multocida, or T. pyogenes; P. multocida and T. pyogenes; and M. bovis and T. pyogenes or H. somni showed co-infection trends. A survey on molecular epidemiology indicated that the occurrence rate of currently prevalent pathogens in BRDC was 46.15% (6/13) for BoHV-1.2b and 53.85% (7/13) for BoHV-1.2c, 53.3% (8/15) for BVDV-1b and 46.7% (7/15) for BVDV-1d, 29.41% (5/17) for BPIV-3c and 70.59% (12/17) for BPIV-3c, 100% (2/2) for BRSV gene subgroup IX, 91.67% (33/36) for P. multocida serotype A, and 8.33% (3/36) for P. multocida serotype D. Our research discovered new subgenotypes for BoHV-1.2c, BRSV gene subgroup IX, and P. multocida serotype D in China’s cattle herds. In the BRDC cases, bovine suppurative or caseous necrotizing pneumonia was highly related to BVDV [odds ratio (OR) = 4.18; 95% confidence interval (95% CI): 1.6–10.7], M. bovis (OR = 2.35; 95% CI: 1.1–4.9), H. somni (OR = 8.2; 95% CI: 2.6–25.5), and T. pyogenes (OR = 13.92; 95% CI: 5.8–33.3). The risk factor analysis found that dairy calves <3 mo and beef calves >3 mo (OR = 5.39; 95% CI: 2.7–10.7) were more susceptible to BRDC. Beef cattle were more susceptible to bovine suppurative or caseous necrotizing pneumonia than dairy cattle (OR = 2.32; 95% CI: 1.2–4.4). These epidemiological data and the new pathogen subgenotypes will be helpful in formulating strategies of control and prevention, developing new vaccines, improving clinical differential diagnosis by necropsy, predicting the most likely pathogen, and justifying antimicrobial use.
Key words: bovine respiratory disease complex, necropsy, pathogen detection, epidemiological investigation

INTRODUCTION

Bovine respiratory disease complex (BRDC) involves a combination of pathogens, stressors, immunologically susceptible animals, and numerous risk factors (Gershwin, 2015; Roland et al., 2016). It is a major cause of morbidity and mortality in feedlot cattle populations and dairy herds, particularly in recently weaned and newly transported calves (Ellis, 2009; Fulton, 2009a; USDA, 2013), and it is responsible for severe economic losses in dairy and feedlot herds worldwide. The pathogens associated with BRDC are highly complex. They mainly include viruses, including bovine herpesvirus-1 (BoHV-1), bovine viral diarrhea virus (BVDV), bovine parainfluenza virus type-3 (BPIV-3), bovine respiratory syncytial virus (BRSV), and bovine coronavirus (BCoV); and bacteria including Mannheimia haemolytica, Pasteurella multocida, and Histophilus somni; occasionally Trueperella pyogenes, and Mycoplasma spp., particularly M. bovis (Ellis, 2009; Griffin et al., 2010; Holman et al., 2015). These pathogens can be further divided into multiple genotypes or serotypes. Bovine herpesvirus-1 is divided into 1.1 and 1.2 (a and b); BVDV is divided into type 1 (21 subtypes), type 2 (4 subtypes), and type 3; BPIV-3 is divided into 3a, 3b, and 3c; BRSV is divided into 8 genetic subgroups (I–VIII); and P. multocida is divided into 5 serotypes (A, B, D, E, and F). Mycoplasma spp. belonging to the Mollicutes class include M. mycoides, M. bovis, M. dispar, M. bovirhinis, M. arginini, and M. alkalescens, of which M. mycoides is the most common (Tortorelli and Carrilo Gaeta, 2017; Kresić et al., 2018; Zeineldin et al., 2019).

The gold standard for BRDC diagnoses is a pathologic postmortem evaluation immediately after the diagnosis of BRDC but, for ethical and economic reasons, this is usually avoided (Wolfger et al., 2015). A postmortem examination and etiological diagnosis are of great value for diagnosing BRDC but cannot be used for early detection (Caswell et al., 2012). The gross lesions in the lungs of cattle with BRDC caused by different pathogens are also diverse. The major types of pneumonia include lobar bronchopneumonia, lobar bronchopneumonia with pleuritis, interstitial pneumonia, broncho interstitial pneumonia, septic pneumonia, pneumonia with embolic foci (bronchopneumonia with multiple foci of caseous necrosis), and suppurrative bronchopneumonia (Fulton et al., 2009b; Caswell et al., 2012). It is difficult for clinical veterinarians or breeders to distinguish between these types of pneumonia, especially with purulent foci or caseous necrosis foci, called bovine suppurrative or caseous necrotizing (BSC) pneumonia or bovine rot pneumonia by clinical veterinarians and breeders.

At present, pathogen detection in BRDC epidemiological research is mostly limited to detecting pathogens in live animals. The sampling methods commonly include nasal swabs, guarded nasopharyngeal swabs, bronchoalveolar lavage, and transtracheal wash, which cannot fully reflect the pathogens responsible for lung tissue lesions (Timsit et al., 2016a; Doyle et al., 2017; Buczinski and Pardo, 2020). In addition to traditional bacterial culture, highly sensitive and specific PCR or reverse transcription (RT)-PCR to detect the pathogens based on lung tissues of BRDC cases resulting in spontaneous death or euthanized animals is increasingly used and can accurately reflect the pathogens associated with lung lesions or death (O’Neill et al., 2014). The diverse factors that can induce BRDC include transport, mixed groups, climate stress, wet and cold environments, dust, dehydration, hypoxia, toxins, and acute metabolic disorders (Zeineldin et al., 2019). However, the relationship between BRDC and risk factors such as climate, age, and breed of susceptible cattle has not yet been reported in China.

Therefore, the objectives of the present study were to reveal the molecular epidemiological characteristics and risk factors of BRDC and BSC pneumonia in Chinese cattle, and to provide theoretical support for the development of new diagnostic methods and vaccines for the prevention and treatment of BRDC.

MATERIALS AND METHODS

Because only postmortem sampling was performed, this study did not require ethical approval.

PCR Primers

The PCR primers for detecting bovine respiratory pathogens (Supplemental Table S1; https://doi.org/10.6084/m9.figshare.21341556) were synthesized by Shanghai Shenggong Biotechnology Service Co. Ltd. The pathogens related to BRDC selected for detection include viruses [BoHV-1, BVDV, BPIV-3, BRSV, BCoV, and bovine parainfluenza virus type-5 (BPIV-5)], bacteria (P. multocida, Mann. haemolytica, H. somni, and T. pyogenes), and several Mycoplasma spp. using universal primers. The PCR typing primers were used to distinguish the A, B, D, E, and F capsular serotypes of P. multocida. Mycoplasma-specific primers were used to distinguish Mycoplasma mycoides spp. mycoides small colony (SC) type, M. dispar, M. bovis, M. bovirhinis, M. arginini, and M. alkalescens.
**Samples and Epidemiological Information**

The samples were collected by the Heilongjiang Key Laboratory of Cattle Diseases and the Animal Hospital of Heilongjiang Bayi Agricultural University from July 2016 to July 2021, in Northeast China, including Heilongjiang Province, Jilin Province, Liaoning Province, and parts of the Inner Mongolia Autonomous Region bordering Heilongjiang and Jilin Provinces. These samples were derived from 81 cattle herds in 33 counties, as shown in Figure 1 and Supplemental Table S2 (https://doi.org/10.6084/m9.figshare.21341556). The 160 lung samples were collected from sick or euthanized cattle with typical bovine respiratory symptoms (e.g., cough, runny nose, difficulty breathing). As far as possible, the samples were collected aseptically through autopsy within 4 h after death. The lung tissue lesions of cattle that died of BRDC were mostly characterized by consolidation and necrosis or suppuration, and most lesions occur in the cardiac and apical lobes. For each case, 2 pieces of lung tissue (4 to 7 cm) were collected aseptically: one was the lobe with consolidation and the other was the junction of consolidation or purulent foci and nonconsolidated lung tissue. Additionally, necropsy lesions were recorded, and samples with autolysis were discarded. According to the characteristics of lung lesions, BRDC cases were divided into BSC pneumonia and “other” pneumonia. The “BSC pneumonia” category is shown in Supplemental Figure S1 (https://doi.org/10.6084/m9.figshare.21341544). The visible lesions of the lungs were diverse in BRDC cases, including fibrinous purulent pneumonia (Figure S1a, b), septic pneumonia (Figure S1c, d), abscesses (Figure S1e), supplicative bronchopneumonia (Figure S1f), interstitial pneumonia with necrotic foci (Figure S1g), and caseous necrosis (Figure S1h, i). Pneumonia with pathological lesions of the lungs described above is called BSC pneumonia.

The “Other pneumonia” category included visible pulmonary fleshy transformation, interstitial pneumonia, lobar pneumonia, lobular pneumonia, and emphysema. The owner or veterinarian supplied epidemiological information on the diseased cattle (including breed, age, and season), as reported in Supplemental Table S3 (https://doi.org/10.6084/m9.figshare.21341556). Most of the BRDC cases in this study were treated with antibiotics, and the herds had not been administered bovine respiratory disease–related vaccines (BoHV-1, BVDV, BPIV-3, BRVS, BCoV, and BPIV-5, *P. multocida*, *T. pyogenes*, *Mann. haemolytica*, *H. somni*, and *Mycoplasma* spp.).
**Detection of Pathogens in Lung Tissue of Dead Cattle with BRDC**

Pathogens from 160 lung tissue samples from cattle that died from BRDC were detected by PCR or RT-PCR using the primers listed in Supplemental Table S1. The viral pathogens included BoHV-1, BVDV, BPIV-3, BRV, BCoV, and BRSV. The bacterial pathogens included *P. multocida*, *T. pyogenes*, *Mann. haemolytica*, *H. somni*, and *Mycoplasma* spp. *Mycoplasma* spp. samples were further analyzed for *M. mycoides* ssp. *mycoides* SC type, *M. dispar*, *M. bovis*, *M. bovirhinis*, *M. arginini*, and *M. alkaelecens* using species-specific PCR. Lung tissue was also used for routine bacterial isolation.

The PCR or RT-PCR was performed as follows. Bovine lung samples were collected and ground aseptically. DNA or RNA was prepared using AP-MN-BF-VNA-250 G AxyPrep humoral virus DNA/RNA preparation kit (Axygen 2617) and ReverTra Ace qPCR RT Master Mix (Toyobo) for cDNA synthesis. The reaction volume was 25 μL and included 12.5 μL of Quick Taq HS DyeMix (Toyobo), 2 μL of cDNA or DNA, 1 μL (12.5 pM) of each pathogen-specific sense and antisense primers, and 8.5 μL of sterile deionized water. The cycling parameters were as follows: 5 min after pre-denaturation at 95°C; followed by 35 cycles of 95°C for 1 min, 56°C (62°C for BoHV-1) annealing temperature for 45 s, 72°C extension for 1 min; and a final extension at 72°C for 10 min. Negative and positive controls were included for each PCR, and the PCR products were analyzed using 1% agarose gel electrophoresis.

During the bacterial test, 2 nutrient agar medium plates with 5% fresh sterile sheep defibrinated blood were inoculated with the aseptically collected lung tissue samples and incubated at 37°C for 48 h under aerobic and CO2 (candle tank method) conditions. The purified bacteria were identified by inspection of bacterial culture characteristics, Gram stain microscopic examination, and identification media (MacConkey and SS medium).

Bacterial 16S rRNA universal primers were used for PCR amplification and sequencing identification of bacteria that the above methods could not identify.

**Isolation and Identification of Virus.** The aseptically collected lung tissue was mixed with Dulbecco’s modified Eagle medium (DMEM) at a ratio of 1:10 and mashed with a tissue masher. The tissue fluid was frozen and thawed twice, centrifuged at 1,000 × g at 4°C for 5 min, and the supernatant was centrifuged at 12,000 × g at 4°C for 15 min. The supernatants were collected, filtered through 0.22-μm filters, and inoculated into Madin-Darby bovine kidney (MDBK) cells at 70 to 80% confluence in a 25-cm² cell culture flask for 1 h before the supernatants were discarded. This was followed by adding 5 mL of DMEM containing penicillin and streptomycin and incubating the flask at 37°C. The inoculated MDBK cells were observed daily for the appearance of cytopathic effect (CPE). When approximately 60 to 70% CPE was observed in MDBK cells, the cultures were collected and subjected to 3 blind passages in MDBK cells. Virus isolated was detected by PCR using the specific primers shown in Supplemental Table S1.

**Phylogenetic Analysis of BoHV-1, BVDV, BPIV-3, and BRSV.** The partial gC gene (575 bp) of BoHV-1, the partial 5’-UTR (untranslated region) sequence (280 bp) of BVDV, the partial M gene (385 bp) of BPIV-3, and the partial G protein gene (848 bp) of BRV were used as the landmark genes for viral genetic evolutionary analysis (Supplemental Table S1). The genes were amplified by PCR or RT-PCR, and the PCR products were bidirectionally sequenced. Bioedit (version 7.0.5.3; http://www.mbio.ncsu.edu/bioedit/bioedit.html) was used to assemble and edit the sequences. The BLAST tool of the National Center for Biotechnology Information (NCBI; https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to compare these sequences with the strain sequences from GenBank. MegAlign software (Thompson et al., 1994) was used for homology analysis of the isolates’ gene sequences and representative reference strains from GenBank. MEGA X software (using the N-J method, Bootstrap was 1000 repeats; Kumar et al., 2018) was used to construct phylogenetic trees to analyze the genetic variation of isolates.

**Serotyping of P. multocida from Lung Tissues**

To examine the *P. multocida* serotypes that are currently prevalent in Chinese cattle herds, *P. multocida* was isolated by culturing lung tissue in 5% fresh sheep blood nutrient agar medium and identified by amplifying part of the kmtl gene (460 bp) with specific primers. The conserved genes *hyaD-hyaC, bchD, dcbF, ecbJ*, and *fcbD* were amplified by PCR with specific primers to differentiate the *P. multocida* capsular serotypes A, B, D, E, and F.
**PCR Typing of Mycoplasma spp. in Lung Tissues**

To examine the species and positivity rate of mycoplasmas in infected lung tissues of dead cattle with BRDC, *Mycoplasma*-positive samples were amplified by PCR using 16S rDNA specific primers of 5 types of mycoplasma, including *M. mycoides* ssp. *mycoides* SC, *M. dispar*, *M. bovis*, *M. bovirhinis*, *M. arginini*, and *M. alkalescens*.

**Characterization of BRDC Pathogenic Infection**

To explore the characteristics of pathogenic co-infection in BRDC cases, the probability of pathogen appearance was analyzed from cases of multipathogen infection in the 160 BRDC cases. The major pathogens detected in BRDC cattle lungs, including BoHV-1, BVDV, BPIV-3, BRSV, *P. multocida*, *T. pyogenes*, *Mann. haemolytica*, *H. somni*, and *M. bovis* were selected for analysis through multiple regression models.

The lung lesions in the 160 BRDC cases could be classified into 67 cases of BSC pneumonia and 93 cases of “other” pneumonia. To explore the pathogens that played a major role in BSC pneumonia, the number of pathogens detected in the 2 types of bovine pneumonia cases from the 160 cases of BRDC was statistically analyzed.

**Analysis of BRDC Risk Factors**

To explore risk factors related to BRDC in Northeast China, we analyzed the effects of age and breed on BRDC. The information was obtained by questioning the owners or attending veterinarians of the 81 cattle herds in which BRDC occurred. Information on sick cattle, including age of birth, breed, month of illness, clinical signs, colostrum management, milk feeding, and transportation, was recorded from the onset of BRDC cases in the herd to the end of the epidemic (including recovery, death, or euthanasia; Supplemental Table S3). The cattle breeds were mainly divided into dairy cattle (Holstein cows) and beef cattle (e.g., Simmental, Charolais, Haifuda, Kobe, Flewich, Australian Wagyu).

**Statistical Analysis**

Data were analyzed using GraphPad Prism 5 (GraphPad Inc.) and SPSS Statistics 26 (IBM Corp.) software. The unit of risk factor analysis was herd. The dairy herd size ranged between 500 and 5,000, and the beef herd size was between 50 and 1,000. Noninterpretable results were not included in the analysis. All statistical comparisons were based on a 5% level of significance. Significance was set at $P < 0.05$, and $P < 0.10$ was considered a trend. Explanatory variables were first examined using univariate models before inclusion in the full multivariate model. All predictors with $P < 0.2$ were maintained for the multivariable model. The risk factors for each of the 10 pathogens were analyzed using multivariable logistic regression (Pardon et al., 2020). The multivariable model was built with a stepwise approach by backward elimination, gradually excluding nonsignificant variables (Fulton et al., 2009; Dubrovsky et al., 2019). The Wilcoxon rank sum test was performed to describe the statistical association between BRDC and age. The specific interactions tested included 2-way interaction terms of BSC pneumonia and “other” pneumonia between weaning age and breeds (Fulton et al., 2009; Dubrovsky et al., 2019). Because the sample sizes were small, percents were compared using Fisher’s exact test. For the final models, pairwise comparisons for categorical predictors were performed using Pearson’s chi-squared test.

**RESULTS**

**Pathogens Detected in Infected Lungs of Cattle with BRDC**

More than 18 species of pathogens, including 491 strains of bacteria, viruses, or *Mycoplasma* species, were detected in 160 lung tissue samples of cattle with BRDC. As shown in Table 1, more than 6 species of bacteria were detected, with a positivity rate of 31.77% (156/491). The pathogen positivity rate was the highest for *T. pyogenes* at 9.37% (46/491), followed by *P. multocida* (8.35%, 41/491). The positivity rate of *Mycoplasma spp.* was 38.9% (191/491). The detection rate of *M. bovis* was 7.74% (38/491) and that of *M. dispar* was 11.61% (57/491). The 6 viruses were detected with a positivity rate of 29.33% (144/491). The pathogen positivity rate was the highest for BoHV-1 at 13.25% (55/491), and BRSV was detected in 5.50% (27/491) of cases. The pathogenic species of BRDC were shown to be diverse in Chinese cattle herds (Supplemental Table S3).

**Infection Characteristics of BRDC Cases**

**Pathogen Infection Rate.** As shown in Table 1, in bacterial infections among the 160 BRDC cases, *T. pyogenes* had the highest positivity rate at 28.75% (n = 46), *P. multocida* accounted for 25.63% (n = 41), *H. somni* for 13.75% (n = 22), and *Mann. haemolytica* for 7.5% (n = 12). The positivity rate of *M. bovis* infection was 23.8% (n = 38). In virus infections, BoHV-1 positivity was the highest at 34.38% (n = 55), followed
Multipathogen Mixed Infection Rate. The multipathogen mixed infection rate is shown in Table 1. The main form of pathogenic infection was a multipathogen mixed infection, accounting for 88.75% (142/160) of cases. The mixed infection ratio of bacteria and other pathogens was 73.75% (n = 118), followed by that of M. bovis and other pathogens (23.75%, n = 38), and the mixed infection rate of viruses and other pathogens was 50% (n = 80). These results indicated that multipathogen mixed infection was the main cause of death of BRDC cattle.

Risk of Pathogen Co-Infection. The risk analysis results for multipathogen co-infection are shown in Table 2. Bovine herpesvirus-1 and H. somni showed a trend toward collaborative infection ($P = 0.0032$, odds ratio (OR) = 4.14). In addition, BVDV and M. bovis ($P = 0.0334$, OR = 2.86), P. multocida ($P = 0.0241$, OR = 2.88), T. pyogenes ($P = 0.0076$, OR = 3.44), and Mann. haemolytica ($P = 0.0031$, OR = 7.22) were associated with co-infection. Moreover, BPIV-3 and M. bovis showed a trend toward collaborative infection ($P = 0.0001$, OR = 7.41), and BRSV and M. bovis ($P = 0.0021$, OR = 4.09), P. multocida ($P = 0.0004$, OR = 5.14), and T. pyogenes ($P = 0.0187$, OR = 2.87) showed a trend toward collaborative infection. Pasteurella multocida and T. pyogenes showed a trend toward collaborative infection ($P = 0.046$, OR = 2.2), and M. bovis with T. pyogenes ($P = 0.0009$, OR = 4.53) and H. somni ($P = 0.0152$, OR = 3.27) showed a trend toward collaborative infection. The above results indicate that the viruses BVDV, BRSV, and BoHV-1 may be the main risk factors for secondary bacterial infection in BRDC, consistent with previous reports (Shahriar et al., 2002; Pardon et al., 2020).

Risk of Pathogen Infection for BSC Pneumonia. Statistical analysis of the pathogens detected in the lung tissue of BRDC cases showed that BSC pneumonia cases in BVDV-positive cases accounted for 70.83% (17/24). This was significantly higher than the 36.76% (50/136) cases of BSC pneumonia in BVDV-negative cases ($P = 0.0029$, OR = 4.18). In M. bovis infection-positive cases, BSC pneumonia cases accounted for 57.89% (22/38), which was significantly higher than the 36.89% (45/122) cases of BSC pneumonia in M. bovis negative cases ($P = 0.025$, OR = 2.35). The cases of BSC pneumonia in H. somni and T. pyogenes positive cases accounted for 72.73% (16/22) and 82.61% (38/46), respectively, significantly higher than cases of BSC pneumonia in H. somni- and T.

### Table 1. Characteristics of pathogenic infection from 160 bovine respiratory disease complex (BRDC) cases in Northeast China between 2016 and 2020

| Pathogen | No. of pathogens or cases | Pathogens/total pathogens, % (n = 491) | Cases/total cases, % (n = 160) |
|----------|--------------------------|----------------------------------------|-------------------------------|
| Bacteria | 156                      | 31.77                                  |                               |
| Pasteurella multocida | 41 | 8.35 | 25.63 |
| Trueperella pyogenes | 46 | 9.37 | 28.75 |
| Mannheimia haemolytica | 12 | 2.44 | 7.50 |
| Histophilus somni | 22 | 4.48 | 13.75 |
| Escherichia coli | 17 | 3.46 | 10.63 |
| Streptococcus | 11 | 2.24 | 6.88 |
| Other bacteria | 7 | 1.43 | 4.38 |
| Mycoplasma spp. | 191 | 38.9 |  |
| M. dispar | 57 | 11.61 | 35.63 |
| M. bovis | 38 | 7.74 | 23.75 |
| M. bovirhinis | 39 | 7.94 | 24.38 |
| M. alkalescens | 30 | 6.11 | 18.75 |
| M. arginini | 4 | 0.81 | 2.50 |
| Undetermined species | 23 | 4.68 | 14.38 |
| Virus | 144 | 29.33 |  |
| Bovine herpesvirus-1 | 55 | 11.20 | 34.38 |
| Bovine viral diarrhea virus | 24 | 4.89 | 15.00 |
| Bovine parainfluenza virus type-3 | 21 | 4.28 | 13.13 |
| Bovine parainfluenza virus type-5 | 6 | 1.22 | 3.75 |
| Bovine respiratory syncytial virus | 27 | 5.50 | 16.88 |
| Bovine coronavirus | 11 | 2.24 | 6.88 |
| Mixed infection | 142 |  | 88.75 |
| Mixed infection of bacterium and other pathogens | 118 |  | 73.75 |
| Mixed infection of virus and other pathogens | 80 |  | 50 |
| Mixed infection of M. bovis and other pathogens | 38 |  | 23.75 |

1 A total of 491 pathogens were detected by PCR or reverse transcription-PCR from lung tissues of 160 BRDC cases, of which 156 bacterial strains accounted for 31.77%, 191 Mycoplasma strains accounted for 38.9%, and 144 viral strains accounted for 29.33%.

2 Percentage of cases with single pathogen infection and mixed infection with different types of single pathogens.

by BRSV at 16.88% (n = 27), BVDV at 15% (n = 24), BPIV-3 at 13.13% (n = 21), BCoV at 6.88% (n = 11), and BPIV-5 at 3.75% (n = 6).

**Multipathogen Mixed Infection Rate.** The multipathogen mixed infection rate is shown in Table 1. The main form of pathogenic infection was a multipathogen mixed infection, accounting for 88.75% (142/160) of cases. The mixed infection ratio of bacteria and other pathogens was 73.75% (n = 118), followed by that of M. bovis and other pathogens (23.75%, n = 38), and the mixed infection rate of viruses and other pathogens was 50% (n = 80). These results indicated that multipathogen mixed infection was the main cause of death of BRDC cattle.

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**Risk of Pathogen Infection for BSC Pneumonia.** Statistical analysis of the pathogens detected in the lung tissue of BRDC cases showed that BSC pneumonia cases in BVDV-positive cases accounted for 70.83% (17/24). This was significantly higher than the 36.76% (50/136) cases of BSC pneumonia in BVDV-negative cases ($P = 0.0029$, OR = 4.18). In M. bovis infection-positive cases, BSC pneumonia cases accounted for 57.89% (22/38), which was significantly higher than the 36.89% (45/122) cases of BSC pneumonia in M. bovis negative cases ($P = 0.025$, OR = 2.35). The cases of BSC pneumonia in H. somni and T. pyogenes positive cases accounted for 72.73% (16/22) and 82.61% (38/46), respectively, significantly higher than cases of BSC pneumonia in H. somni- and T.
To explore the risk factors affecting the occurrence of BRDC in Northeast China, we performed a cross-sectional epidemiological analysis of the 81 cattle herds submitted for inspection from July 2016 to July 2021. The positive rate of farms (P) was used to assess the association of BRDC with cattle age:

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P = \frac{\text{No. of farms with diseased cattle at corresponding age}}{\text{Total no. of cattle farms}} \times 100%.
\]

Table 3. Pathogenic risk factors of 67 cases of bovine suppurative or caseous necrotizing (BSC) pneumonia in 160 bovine respiratory disease complex (BRDC) cases

| Independent variable | Category | BSC pneumonia, % (n/m)\(^1\) | P-value | Odds ratio | 95% CI |
|----------------------|----------|-------------------------------|---------|-----------|--------|
| Bovine herpesvirus-1 | Positive | 49.09 (27/55)                | 0.2375  | 1.57      | 0.81–3.03 |
|                      | Negative | 38.1 (40/105)                |         |           |        |
| Bovine viral diarrhea virus | Positive | 70.83 (17/24) | 0.0029 | 4.18 | 1.62–10.77 |
|                      | Negative | 36.76 (50/136)               |         |           |        |
| Bovine parainfluenza virus type-3 | Positive | 33.33 (7/21) | 0.4805 | 0.66 | 0.25–1.73 |
|                      | Negative | 43.17 (60/139)               |         |           |        |
| Bovine respiratory syncytial virus | Positive | 25.93 (7/27) | 0.0867 | 0.43 | 0.17–1.08 |
|                      | Negative | 45.11 (60/133)               |         |           |        |
| Mycoplasma bovis     | Positive | 57.89 (22/38)                | 0.0250  | 2.35      | 1.12–4.94 |
|                      | Negative | 36.89 (45/122)               |         |           |        |
| Histophilus somni    | Positive | 72.73 (16/22)                | 0.0001  | 8.17      | 2.62–25.52 |
|                      | Negative | 36.96 (51/138)               |         |           |        |
| Pasteurella multocida| Positive | 51.22 (21/41)                | 0.1992  | 1.67      | 0.82–3.41 |
|                      | Negative | 38.66 (4/119)                |         |           |        |
| Mannheimia haemolytica| Positive | 50 (6/12)                    | 0.5596  | 1.43      | 0.44–4.63 |
|                      | Negative | 41.23 (61/148)               |         |           |        |
| Trueperella pyogenes | Positive | 82.61 (38/46)                | 0.0001  | 13.92     | 5.82–33.28 |
|                      | Negative | 25.44 (29/114)               |         |           |        |

\(^1\)Where n = number of BSC pneumonia cases and m = number of BRDC cases.
As shown in Figure 2, the positive rate of farms of 0- to 1-mo-old, 1- to 2-mo-old, and 2- to 3-mo-old calves with BRDC was higher than that at other ages. Especially, 1- to 2-mo-old calves had the highest rate (58.02%), which was significantly higher than that of 2- to 3-mo-old calves ($P < 0.05$). By 8 mo of age, BRDC incidence decreased significantly ($P < 0.01$) to 8.6% (7/81).

The positivity rate of BRDC in dairy herds was 69.11% (47/68) from 0 to 3 mo of age, which was significantly higher than that of beef herds at 30.88% ($P < 0.05$). By 8 mo of age, BRDC incidence decreased significantly ($P < 0.01$) to 8.6% (7/81).

In BRDC cases, the probability of BSC pneumonia occurring in beef cattle was 51.16% (44/86), significantly higher than the probability of BSC pneumonia in dairy cows at 31.08% (23/74) ($P = 0.0156$, $OR = 5.388$). Thus, dairy cattle are more likely to develop BRDC than Holstein dairy cattle (Table 4).

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**Molecular Epidemiological Survey of Pathogens from BRDC Cases**

*Mycoplasma spp.* To determine the species of *Mycoplasma* in BRDC cases, the 103 *Mycoplasma*-positive samples were classified by PCR using specific primers for the 6 bovine *Mycoplasma* spp. that infect the bovine respiratory tract. As shown in Table 1, 191 *Mycoplasma* strains were detected; *M. dispar* had the highest detection rate of 29.84% (57/191), followed by *M. bovirhinis* (20.42%, 39/191). In contrast, the detection rate of traditionally highly pathogenic *M. bovis* was only 19.9% (38/191). In addition, *M. alkalesscens* was positive in 15.71% (30/191) and *M. arginini* was positive in 2.09% (4/191) of cases. *Mycoplasma mycoides* ssp. *mycoides* was not detected in any of the samples. In addition, the *Mycoplasma*-positive samples that could not be typed accounted for 12.04% (23/191) of cases. *Mycoplasma mycoides* ssp. *mycoides* was not detected in any of the samples. In addition, the *Mycoplasma*-positive samples that could not be typed accounted for 12.04% (23/191) of cases. *Mycoplasma mycoides* ssp. *mycoides* was not detected in any of the samples. In addition, the *Mycoplasma*-positive samples that could not be typed accounted for 12.04% (23/191) of cases. *Mycoplasma mycoides* ssp. *mycoides* was not detected in any of the samples.

53.9% (61/113) of the *Mycoplasma* strains were classified into the BoHV-1.2 gene subtype but located in 2 phylogenetic tree branches with a long genetic distance (Figure 4). One of the 2 branched BoHV-1 strains accounted for 46.15% (6/13) of the BoHV-1.2b gene subtype. The other branch BoHV-1 strains accounted for 53.85% (7/13), which was different from the traditional BoHV-1.2a and -1.2b gene subtype, and was named the BoHV-1.2c gene subtype, as described recently by our group (Zhou et al., 2020).

**Isolation, Identification, and Genetic Evolution Analysis of BoHV-1**

Among the 55 BoHV-1-positive samples inoculated on MDBK cells, 42 samples showed BoHV-1 typical CPE (grape string or aggregation) after 1 to 3 passages, and the isolated BoHV-1 was determined by PCR amplification of the 339-bp gB gene fragment. The 13 BoHV-1 isolates with regional representation were selected to amplify the gC gene for genetic evolution analysis. According to the phylogenetic analysis of a 451-bp gC gene fragment, all 13 BoHV-1 isolates were classified into the BoHV-1.2 gene subtype but located in 2 phylogenetic tree branches with a long genetic distance (Figure 4). One of the 2 branched BoHV-1 strains accounted for 46.15% (6/13) of the BoHV-1.2b gene subtype. The other branch BoHV-1 strains accounted for 53.85% (7/13), which was different from the traditional BoHV-1.2a and -1.2b gene subtype, and was named the BoHV-1.2c gene subtype, as described recently by our group (Zhou et al., 2020).

**Isolation, Identification, and Genetic Evolution Analysis of BVDV**

The MDBK cells were inoculated with 24 BVDV-positive lung samples identified by RT-PCR. After the third blind passage, 15 BVDV isolates were identified by RT-PCR using the 5′-UTR specific primer; 12 BVDV isolates were of the cytopathic (CP) biotype (cell lengthening, stringing, intercellular space enlargement), and 3 BVDV isolates were noncytopathic (NCP) biotypes. The 288-bp 5′-UTR products of the 15 BVDV isolates were sequenced, and a 250-bp gene sequence was used for genetic evolution analysis. As shown in Figure 5, 2 BVDV-1 subtypes were detected: BVDV-1b was detected in 53.3% (8/15) and BVDV-1d in 46.7% (7/15) of cases.

**Isolation, Identification, and Genetic Evolution Analysis of BPIV-3**

Among the 21 BPIV-3-positive lung samples used to inoculate the MDBK cells, 17 showed BPIV-3 typical CPE (cell swelling, rounding, shedding, and cell fusion) after 1 to 3 passages. The 330-bp BPIV-3 M gene fragment from the 17 BPIV-3 isolates was amplified by RT-PCR and sequenced for genetic evolution analysis. The results revealed 2 genotypes in these BPIV-3 isolates:
Figure 2. (a) Statistical heat graph representing significant differences in the positive rate of farms with bovine respiratory disease complex (BRDC) and (b) bar chart representing the positive and negative rate of farms with BRDC in calves of different ages.
BPIV-3a was found in 29.41% (5/17) and BPIV-3c was observed in 70.59% (12/17) of cases (Figure 6). Another genotype, BPIV-3b, was not detected in this study.

**Genetic Evolution Analysis of BRSV**

In this study, 2 lung tissue samples with typical BRSV infection lesions from 2 cattle herds with high mortality caused by BRDC in Qiqihar City (dairy cattle herd) and Anda City (beef cattle herd) were identified as BRSV positive by RT-PCR and used for BRSV G gene sequencing. The 848-bp gene fragment of the BRSV G gene was obtained by reverse transcription and semi-nested PCR (Valentova et al., 2005), and the 750-bp G gene fragments from the 2 samples were sequenced for genetic evolution analysis. The similarity of the G gene sequence was 98.8% between the 2 BRSV strains and was 85.3 to 89.7% among the 2 strains and the gene types I–VIII of BRSV strains from GenBank (Table 6). In the phylogenetic analysis, the 2 BRSV strains were located on the same branch, independent of the previously reported genotypes (Figure 7) and were tentatively named genotype IX. These results indicated that BRSV shows clear genetic variation in Northeast China.

**DISCUSSION**

The etiology of BRDC is complicated and involves many types of pathogens infecting the bovine respiratory tract, including viruses, bacteria, and *Mycoplasma* spp. (Griffin et al., 2010; Alexander et al., 2020). Pathological necropsy and pathogen detection in deaths from acute or chronic BRDC can reflect BRDC pathogen infection characteristics. Few epidemiological studies are based on detecting pathogens from lung samples because of the difficulty in collecting lung tissue samples from dead cattle. Given this, we sampled 160 lungs from cattle that died from BRDC collected from 2016 to 2021 as pathogen detection samples. Our results revealed that 3 times more pathogens (491 strains) than the number of cases (160 cases) were detected, which shows that multi-pathogen mixed infection [88.75% (142/160) of cases] was the leading cause of death in BRDC. These findings suggested that the prevention and treatment of BRDC should include comprehensive measures against bacteria, viruses, and *Mycoplasma* to achieve the desired effect, and that it is important to develop a combined vaccine to prevent BRDC. Considering that the 160 lung samples in this study were all from BRDC cases resulting in natural deaths (animals that were in the late stage of the disease and had a long disease course), some of the primary viral pathogens infected in the early stage would have disappeared, whereas opportunistic pathogens proliferated in large numbers. Thus, the type and quantity of viral pathogens detected in this study might be lower than that during actual infection, whereas the detection rate of

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### Table 4. Correlation analysis of age and breed (dairy vs. beef breeds) in 160 bovine respiratory disease complex cases

| Age  | Dairy cattle, % (n1/m1) | Beef cattle, % (n2/m2) | P-value | Odds ratio | 95% CI |
|------|------------------------|------------------------|---------|------------|-------|
| 0–3 mo | 69.11 (47/68) | 30.88 (21/68) | <0.0001 | 5.39 | 2.72–10.67 |
| >3 mo | 29.35 (27/92) | 70.65 (65/92) |         |          |       |

1Where n1 = number of dairy cattle; n2 = number of beef cattle; m1 = number of cattle <3 mo; m2 = number of cattle >3 mo.

### Table 5. Correlation analysis between the breeds and bovine suppurative or caseous necrotizing (BSC) pneumonia in 160 bovine respiratory disease complex (BRDC) cases

| Variable | BSC pneumonia, % (n/m) | P-value | Odds ratio | 95% CI |
|----------|------------------------|---------|------------|-------|
| Beef cattle | 51.16 (44/86) | 0.0156 | 2.32 | 1.21–4.44 |
| Dairy cattle | 31.08 (23/74) |         |          |       |

1Where n = number of BSC pneumonia cases; m = number of BRDC cases.

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**Figure 3.** Characteristics of individual infection or co-infection of *Mycoplasma* species in 80 bovine respiratory disease complex (BRDC) cases in which specific *Mycoplasma* species were detected.
opportunistic pathogens exceeds the infection rate in the early stage of the disease. In addition, these cases were treated with antibiotics, which could have resulted in the suppression of certain antibiotic-susceptible bacteria or mycoplasma pathogens. Because the main pathogenic bacteria in BRDC are aerobic bacteria, *M. bovis*, *T. pyogenes*, and *H. somni* do not need to be cultured under strict anaerobic conditions, so strict anaerobic culture was not performed in this study. There are likely more anaerobes present than the most common pathogens of BRDC, and the lack of strict anaerobic culture was a weakness of this study.

The primary infectious pathogens in the BRDC epidemic were BoHV-1, BVDV, BRSV, and BPIV-3. The positivity rate of BoHV-1 in the lungs of BRDC cases was the highest at 34.38% (Table 2), which is higher than the positivity rate (24.83%) of nasal swabs from animals with BRDC living in Inner Mongolia, China, which may be related to differences in the tested samples from different sources (Guo et al., 2021). It is important to note that a new subgenotype of BoHV-1, BoHV-1.2c, was highly endemic to Northeast China, accounting for 53.85% (7/13) of cases. In China, BVDV strains in cattle herds are highly diverse, including 2 genotypes and many subgenotypes (BVDV-1a, b, c, d, m, p, q, u, and BVDV-2a; Hou et al., 2019). However, BVDV-1b (50%, 8/16) and BVDV-1d (50%, 8/16) were the dominant epidemic strains in the BRDC cases in Northeast China. The BVDV-1b subgenotype is epidemic in almost all countries where sequence data exist; BVDV-1d is prevalent in many countries, including China, Germany, Italy, Poland, Turkey, Slovenia, Austria, and Japan (Yeşilbağ et al., 2017). Thus, we speculate that BVDV-1b and 1d are also the main genotypes currently prevalent in Chinese cattle herds. Bovine parainfluenza virus type-3 has 3 subgenotypes (a, b, and c) and only BPIV-3a and BPIV-3c were detected in Northeast China, of which BPIV-3c was the dominant endemic strain with a positivity rate of 70.59% (12/17). The well-understood sensitivity of BRSV has resulted in inefficient virus isolation from clinical samples (Valentova et al., 2005). We attempted to isolate BRSV from clinical samples but were unsuccessful. Thus, 2 representative BRSV RT-PCR-positive samples were selected for G gene sequencing for molecular epidemiological investigation. According to the genetic evolution analysis of the BRSV G gene as shown in Figure 7, the 2 BRSV strains detected in this study could not be classified to the one of the gene subgroups I to VIII previously described (Valentova et al., 2005; Krešić et al., 2018). Therefore, they were temporarily designated as belonging to novel gene sub-
group IX. This is important for further research on the genetic evolution and pathogenic mechanisms of BRSV. As shown in Supplemental Figure S1g (https://doi.org/10.6084/m9.figshare.21341544), the BRSV AND A strain, M. bovis, M. dispar, and P. multocida serotype A were simultaneously detected in lung tissue of cattle with BRDC, which might be due to the immunosuppressive effect caused by BRSV increasing the risk of infection by other pathogens (Gershwin, 2007). The lungs showed clear emphysema, fleshy lesions,
suppurative foci, caseous necrosis foci, and fibrinous pneumonia pathological lesions. Lung emphysema was also seen in bovine lungs infected with the BRSV QQHER strain and other BRSV strains in previous reports (Gershwin, 2007). Therefore, lung emphysema can be a characteristic of severe cases of BRSV. This study explored the association among co-infected pathogens in the lungs of BRDC cases. To meet the statistical requirements, co-infection by 2 pathogens was not analyzed when <6 cases were identified. Co-infecting pathogens without significant differences are not listed in Table 2. As shown in Table 2, the viruses BVDV, BRSV, and BoHV-1 were the main risk factors for secondary bacterial infection in BRDC. This might be related to the disturbance of the host’s immune system, including breakdown of the upper respiratory

**Figure 6.** Phylogenetic analysis of bovine parainfluenza virus type-3 (BPIV-3) based on 330 nucleotides of partial M gene. The tree included 17 BPIV-3 isolates (●) and 3 reference strain sequences from GenBank (Supplemental Table S4; https://doi.org/10.6084/m9.figshare.21341556).

**Table 6.** Percentage of similarity generated by pairwise comparison of G gene sequences of bovine respiratory syncytial virus (BRSV) isolates and different BRSV genotypes

| Virus strain | GenBank accession no. | Classification1 | Nucleotide sequence similarity2 |
|--------------|-----------------------|-----------------|---------------------------------|
| 4642         | Y08718                | I               | ANDA 85.7 QQHER 85.7            |
| BovX         | U57823                | I               | ANDA 85.5 QQHER 85.5            |
| 8307027      | BRU92098              | II              | BovX 87.5 ANDA 87.5             |
| FV160        | AF188578              | II              | ANDA 87.5 BovX 87.5             |
| BRSATTGLYF   | L08415                | III             | BRSATTGLYF 89.7 ANDA 89.7      |
| 391.2        | M58307                | III             | 391.2 87.5 ANDA 87.3            |
| SNOOK        | Y08719                | IV              | SNOOK 88.1 ANDA 88.1            |
| Dorset       | BRU24715              | IV              | ANDA 87.5 Dorset 87.5           |
| 88P          | AF188604              | V               | 88P 86.9 ANDA 86.9              |
| 58P          | AF188603              | V               | 58P 87.1 ANDA 87.1              |
| 75P          | AF188587              | VI              | 75P 87.7 ANDA 87.7              |
| K1           | AF188585              | VI              | K1 87.3 ANDA 87.3               |
| B29/12/CRO   | KY680321              | VII             | B29/12/CRO 85.3 ANDA 85.7      |
| B26/12/CRO   | KY680320              | VII             | B26/12/CRO 85.3 ANDA 85.7      |
| B60279/2/14/CRO| KY680330            | VIII            | B60279/2/14/CRO 87.5 ANDA 87.5 |
| B10152/2/16/CRO| KY680334           | VIII            | B10152/2/16/CRO 86.3 ANDA 86.3 |
| A2           | M11486                | HRSV            | ANDA 54.4 A2 54.6              |
| ANDA         | OM172493              | IX              | ANDA 100 QQHER 98.8            |

1References, I–VI: Krešić et al, 2018 and Valarcher et al, 2000; VII–IX: Krešić et al, 2018.
2BRSV isolates ANDA and QQHER from Heilongjiang Province, China.
tract mucosa, imbalance of flora, and immunosuppression caused by persistent viral infection through immune evasion (Gershwin, 2007; Srikumaran et al., 2007; Jones, 2019). Cattle infected with BVDV appeared predisposed to bacterial pneumonia, especially to co-infections with *P. multocida* (*P* = 0.02, OR = 2.88), *Mann. haemolytica* (*P* < 0.01, OR = 7.22), *T. pyogenes* (*P* < 0.01, OR = 3.44), and *M. bovis* (*P* = 0.03, OR = 2.86), which is consistent with previous reports (Caswell et al., 2010).

Autopsies of cattle with BRDC showed that the lung lesions were complex and diverse; BSC pneumonia cases accounted for 41.88% (67/160; Table 3) of cases. The lung lesions were characterized by caseous necrosis caused by *M. bovis* infection, purulent pneumonia caused by *T. pyogenes* infection, and fibrinous and purulent pneumonia caused by *P. multocida*, *Mann. haemolytica*, or *H. somni* (Caswell et al., 2010; Griffin et al., 2010; Bassel and Caswell, 2018).

Because the above-mentioned pathogens are often present as co-infections resulting in atypical lung lesions, we referred to this type of pneumonia as “BSC pneumonia.” The BSC pneumonia is a characteristic type of chronic pneumonia associated with BRDC and is mostly caused by bacteria that infect the lungs and induce leukocytes to migrate to the lungs. In contrast, neutrophils clear pathogens through oxidative bursts, phagocytosis, or neutrophil extracellular traps (NETs; Brinkmann et al., 2004; Bassel and Caswell, 2018). Neutrophils have protective bactericidal effects but also induce tissue damage by releasing proteases, oxygen radicals, or cytotoxic effects (Bassel and Caswell, 2018).

An important finding of the current study was the incidence that BVDV, *M. bovis*, *H. somni*, and *T. pyogenes* infection was significantly higher in BSC pneumonia than in other types of pneumonia (Table 3), suggesting that *M. bovis*, *H. somni*, and *T. pyogenes* might be secondary infections of BVDV in BSC pneumonia. Primary BVDV infection causes neutropenia and is a risk factor for secondary infection of bacterial pneumonia. This is also common in cattle with caseonecrotic bronchopneumonia (Shahriar et al., 2002; Gagea et al., 2006; Keller et al., 2006). Bovine viral diarrhea virus can cause leukopenia and lymphopenia in the early stages of infection and cause a significant decrease in phagocytosis and killing of neutrophils within 2 wk after infection (Gånheim et al., 2005). Moreover, cytopathic or noncytopathic BVDV-infected neutrophils

**Figure 7.** Phylogenetic analysis of bovine respiratory syncytial virus (BRSV) based on 750 nucleotides of partial G gene. The tree included 2 BRSV strains (●) and 17 reference strain sequences from GenBank (Supplemental Table S4; https://doi.org/10.6084/m9.figshare.21341556).
lead directly to a decrease in the expression of CD18 and CD62L, thereby inhibiting neutrophils from exuding blood vessels and migrating to tissues (Thakur et al., 2014). It also leads to increased expression of CD14, which mainly recognizes LPS from the cell wall of gram-negative bacteria (Thakur et al., 2014). Lipopolysaccharide binds to the CD14 receptor of neutrophils, leading to increased production of pro-inflammatory factors tumor necrosis factor-α and IL-1 (Sohn et al., 2007; Molina et al., 2014). Therefore, we speculate that secondary bacterial infection may be due to BVDV infection causing early leukopenia in the body, inhibiting the migration of neutrophils to lung tissue with bacterial infection, reducing the killing function of neutrophils, and promoting the pro-inflammatory reaction caused by gram-negative bacteria.

In this study, P. multocida and Mann. haemolytica showed no significant difference in infection rates between BSC and “other” pneumonia. This may be because antibiotics are widely used to treat BRDC and the bacteria may be suppressed (Griffin et al., 2010). Worldwide, P. multocida serogroup A isolates are a major cause of BRDC (Dabo et al., 2007; Ma et al., 2010). In this study, 2 P. multocida serotypes were detected; serotype A was detected in 91.67% (33/36), and serotype D in 8.33% (n = 3/36) of cases. Serotype D is associated with atrophic rhinitis and pneumonia in pigs and with avian cholera. In this study, serotype D was first detected in cattle with BRDC. The 3 serotype D strains were isolated from the same Holstein herd in July 2019 and September 2020. Two serotype D strains were derived from the calves that died from BRDC aged 30 to 60 d and exhibited fibrinous purulent pneumonia. The other strain was isolated from a cow that dies suddenly at 20 d postpartum and exhibited fibrinous purulent pneumonia. Thus, P. multocida serotype D can infect both calves and adult cattle. It has become a prevalent strain in a bovine herd in Northeast China and is a possible pathogen of fibrinous purulent pneumonia and fibrinous necrosis bronchial pneumonia.

Two forms of lung lesions were observed in BRDC cases when T. pyogenes was isolated. One was pleural effusion, where the surface is covered with fibrin, the alveolar collapses and shrinks, and the bronchioles or alveoli are fused with purulent foci with pus inside (Supplemental Figure S1a). The other was where pustules of different sizes were apparent in most areas of the cardiac lobe and apical lobe of the lung with connective tissue cysts outside, and the contents became a viscous paste or liquid (Supplemental Figure S1e). However, pleural effusion was not observed. All T. pyogenes infection cases were multipathogen infections. Trueperella pyogenes is regarded as a secondary infectious bacterium in BRDC, and its pathogenic effects have been neglected (Griffin et al., 2010). However, in this study, the bacterial species with the highest detection rate was T. pyogenes, which reached 28.75% (46/160). Fulton et al. (2009b) also showed that T. pyogenes had the highest detection rate (35.0%) among lung tissues of cattle with BRDC. Pneumonia caused by T. pyogenes has rarely been reported in animals such as pigs and poultry (Rzewuska et al., 2019). Trueperella pyogenes isolated from lung tissues of BRDC cases successfully infected goats and created a purulent pneumonia model, suggesting that the pathogenic role of T. pyogenes in BRDC is of concern (Sun et al., 2018).

Mycoplasma bovis is most commonly considered the cause of chronic caseonecrotic bronchopneumonia with or without arthritis (Caswell et al., 2012). In addition, M. bovis is increasingly recognized as a primary pathogen, although this remains controversial (Calcutt et al., 2018). However, the economic importance of M. bovis cannot be accurately measured at present because the clinical signs caused by M. bovis are not specific, and some forms of lung lesions caused by M. bovis cannot be easily distinguished from those caused by other bacteria (Caswell et al., 2012). Our results showed that all 38 cases of M. bovis infection were co-infected with other pathogens (Table 1), confirming that the co-infection of M. bovis with other respiratory pathogens is a common occurrence (Shahriar et al., 2002; Gagea et al., 2006). Moreover, primary pathogens such as BoHV-1, BVDV, BPIV-3, or BRSV were detected in all 38 cases of M. bovis infection, and T. pyogenes, H. somni, P. multocida, M. dispar, or E. coli were detected in 94.7% (36/38) of M. bovis infection cases. Based on the above, we presumed that M. bovis is a secondary pathogen in Chinese BRDC that can promote bacterial infection of the bovine respiratory tract. We also found that co-infection among members of Mycoplasma spp. is very common. Overall, 25.5% (n = 26) of cases had 2 types of mycoplasmas, and 2.91% (n = 3) of cases had co-infection with 5 types of mycoplasmas (Figure 3). The mycoplasma with the highest detection rate in BRDC cases in this study was M. dispar (35.63%, n = 57), not M. bovis (23.75%, n = 38). In addition, in a few typical caseonecrotic lesions of the lungs, either M. dispar alone was detected or M. bovis was detected together with M. dispar. In our investigation, M. dispar was regularly isolated from bovine pnemonic lungs, and its presence was associated with mild infection. In a previous study, M. dispar was one of the most important causes of respiratory disease in cattle (Tortorelli and Carrillo Gaeta, 2017). Many studies have indicated that M. dispar is present in 50% of examined bovine herds and that it coexists with other bacterial agents, such as P.
multocida, T. pyogenes, and Mann. haemolytica (Bednarek et al., 2012). Our study showed that M. dispar is an important pathogen in BRDC in Chinese herds and may play a role in the pathogenicity of pathogens associated with BRDC in a form similar to that of M. bovis. Mycoplasma dispar and M. bovirhinis are the most abundant species in the bovine nasopharynx, accounting for 53% of the total bacterial population (Timits et al., 2016a). Although M. bovirhinis is one of the most commonly occurring species in bovine respiratory diseases (Ayling et al., 2004), it is not considered a primary pathogen because it is frequently isolated from healthy or asymptomatic animals and it might be part of the natural bacterial flora. Mycoplasma hyorhinis usually infects swine, leading to respiratory tract disease and inflammation of the chest and joints (Kobisch and Friis, 1996). In addition, accumulating evidence suggests that M. hyorhinis infection in humans results in clinical outcomes (Huang et al., 2001). The positivity rate of M. bovirhinis was 24.38% (n = 39) in lung tissue of dead cattle with BRDC, suggesting that it may also be an important pathogen of BRDC (Griffin et al., 2010). Undetermined mycoplasma species, except for M. mycoides ssp. mycoides SC, M. bovis, M. dispar, M. bovirhinis, M. alkalensis, and M. arginini, were observed in 14.38% (n = 23) of cases of this study, showing that there are unidentified mycoplasma species in the lungs of cattle with BRDC. To formulate an effective method to control Mycoplasma infection, it is important to conduct an in-depth study of the pathogenicity of different mycoplasma species in BRDC. The gross lesions of lung tissue are similar between BSC pneumonia and bovine tuberculosis. Mycobacterium bovis was not detected in BSC pneumonia cases by histology, Neelsen staining, or PCR methods in a previous study (Guo et al., 2010; Yao et al., 2016; ). However, M. bovis was not tested in the current study, which is a weakness in the study.

Risk factors for BRDC have been demonstrated in groups comprising animals from multiple sources, including host genetics, mode of delivery, diet and the microbiota of the mother, environmental housing, weaning, feeding type, transportation, commuting, antibiotic treatment, vaccination, and pathogen exposure (Zeineldin et al., 2019). We found that the incidence of BRDC in calves ≤3 mo was significantly higher than that in older calves, and the highest BRDC positivity rate was 58%, between 1 and 2 mo of age. This is consistent with the results of a BRDC study of American dairy calves (USDA, 2012; Dubrovsky et al., 2019). Under the current feeding model in China, beef calves rely on cows to feed them until they are weaned at 3 mo; dairy calves are fed milk replacement products and generally weaned at 2 mo. Importantly, weaning stress can also cause BRDC development. Therefore, the reasons described above may cause the incidence rate around 3 mo of age to be significantly higher than that at other ages.

Breed was identified as an important risk factor for BRDC. Previous studies have provided evidence of the association between breed and BRDC. A reasonable biological approach has been proposed, as genetic susceptibility has been shown to vary between breeds (Snowder et al., 2006; Neibergs et al., 2011). Table 4 shows that dairy cows from 0 to 3 mo were more susceptible to BRDC than beef cattle of the same age, whereas beef cattle >3 mo were more susceptible to BRDC than dairy cows of the same age (P < 0.0001, OR = 5.39). In addition, the incidence of dairy cattle suffering from BSC pneumonia was significantly lower than that of beef cattle (P = 0.0156, OR = 2.32). This may be because beef cattle have to be transferred to the fattening field after weaning and experience more severe weaning, transportation, and mixed-group stress than dairy cows. As the age of the calf increases, its immune system matures, and its resistance to pathogens increases, leading to chronic BRDC cases characterized by BSC pneumonia. These observations showed a correlation between the breed and BRDC, most likely due to differences in feeding and management methods in different cattle breeds.

Bovine respiratory disease complex is a multifactorial syndrome involving multiple pathogens, and it is affected by multiple nonpathogenic factors, such as the host, surrounding environment, and management practices (Buckham Sporer et al., 2008; McMullen et al., 2019). When cattle encounter different stressors or are infected with bovine respiratory viruses (BoHV-1, BVDV, BRV, and BPIV-3), their defense ability is weakened. This leads to nasopharyngeal dysbiosis causing abnormal proliferation of pathogenic commensal bacteria, such as Mann. haemolytica, H. somni, P. multocida, T. pyogenes, M. bovis, M. dispar, Ureaplasma diversum, and M. bovirhinis that colonize the upper respiratory tract mucosa and invade the lungs via inhalation (Fulton et al., 2009; Griffin et al., 2010; Timits et al., 2020).

CONCLUSIONS

In the cattle herds of Northeast China, mixed infection with more than 2 pathogens is a clear feature of BRDC; the common pathogens include BoHV-1, T. pyogenes, P. multocida, and M. bovis; BoHV-1.2c, BVDV-1b and 1d, BPIV-3c, BRV gene subgroup IX, and serotype A of P. multocida are the popular subgenotypes. Co-infection with multiple mycoplasmas or between mycoplasmas and bacteria or viruses is common in cattle...
with BRDC. In the case of BSC pneumonia, the main pathogens are BVDV-1, *M. bovis*, *T. pyogenes*, and *H. somni*. Beef cattle are more susceptible to BSC pneumonia than dairy cattle. These data have important guiding significance for the development of multivalent vaccines for the prevention and control of BRDC.

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**REFERENCES**

Alexander, T. W., E. Timsit, and S. Amat. 2020. The role of the bovine respiratory bacterial microbiota in health and disease. Anim. Health Res. Rev. 21:168–171. https://doi.org/10.1017/S1466252320000316.

Aylng, R. D., S. E. Bashiruddin, and R. A. Nicholas. 2004. *Myco- plasma* species and related organisms isolated from ruminants in Britain between 1990 and 2000. Vet. Rec. 155:413–416. https://doi.org/10.1136/vr.155.16.413.

Bassel, L. L., and J. L. Caswell. 2018. Bovine neutrophils in health and disease. Cell Tissue Res. 371:617–637. https://doi.org/10.1007/s00441-018-2789-y.

Bednarz, D., M. Szymańska-Czerwińska, and K. Dudek. 2012. Bovine respiratory syndrome (BRD) etiopathogenesis, diagnosis and control. Pages 363–378 in A Bird’s-Eye View of Veterinary Medicine. C. C. Perez-Marin, ed. InTech.

Brinkmann, V., U. Reichard, C. Goosmann, B. Fauler, Y. Uhlemann, D. S. Weiss, Y. Weinrauch, and A. Zychlinsky. 2004. Neutrophil extracellular traps kill bacteria. Science 303:1532–1535. https://doi.org/10.1126/science.1092385.

Buckham Sporer, K. R., P. S. Weber, J. L. Burton, B. Earley, and M. A. Crowe. 2008. Transportation of young beef bulls alters circulating physiological parameters that may be effective biomarkers of stress. J. Anim. Sci. 86:1325–1334. https://doi.org/10.2527/jas.2007-0762.

Buczynski, S., and B. Pardom. 2020. Bovine respiratory disease diagnosis: What progress has been made in clinical diagnosis? Vet. Clin. North Am. Food Anim. Pract. 36:399–423. https://doi.org/10.1016/j.cvfa.2020.03.001.

Calcutt, M. J., I. Lysnyansky, K. Sachse, L. K. Fox, R. A. J. Nicholas, and R. D. Ayling. 2018. Gap analysis of *Mycoplasma bovis* disease, diagnosis and control: An aid to identify future development requirements. Transbound. Emerg. Dis. 65(Suppl. 1):91–109. https://doi.org/10.1111/tbed.12800.

Caswell, J. L., K. G. Bateman, H. Y. Cai, and F. Castillo-Alcala. 2010. *Mycoplasma bovis* in respiratory disease of feedlot cattle. Vet. Clin. North Am. Food Anim. Pract. 26:365–379. https://doi.org/10.1016/j.cvfa.2010.03.003.

Caswell, J. L., J. Hewson, D. Slávčík, J. DeLay, and K. Bateman. 2012. Laboratory and postmortem diagnosis of bovine respiratory disease. Vet. Clin. North Am. Food Anim. Pract. 28:419–441. https://doi.org/10.1016/j.cvfa.2012.07.004.

Dabo, S. M., J. Taylor, and A. Confer. 2007. *Pasteurella multocida* and bovine respiratory disease. Anim. Health Res. Rev. 8:129–150. https://doi.org/10.1017/S1466252307001399.

Doyle, D., B. Credille, T. W. Lehenbauer, R. Berghaus, S. S. Aly, J. Champagne, P. Blanchard, B. Crossley, L. Berghaus, S. Cochran, and A. Woolums. 2017. Agreement among 4 sampling methods to identify respiratory pathogens in dairy calves with acute bovine respiratory disease. J. Vet. Intern. Med. 31:954–959. https://doi.org/10.1111/jvim.14083.

Dubrovskev, S. A., A. L. Van Eenennaam, B. M. Karle, P. V. Rossitto, T. W. Lehenbauer, and S. S. Aly. 2019. Bovine respiratory disease (BRD) cause-specific and overall mortality in preweaned calves on California dairies: The BRD 10K study. J. Dairy Sci. 102:7320–7328. https://doi.org/10.3168/jds.2018-15463.

Ellis, J. A. 2009. Update on viral pathogenesis in BRD. Anim. Health Res. Rev. 10:141–193. https://doi.org/10.1017/S146625230999020X.

Fulton, R. W. 2009. Bovine respiratory disease research (1985–2009). Anim. Health Res. Rev. 10:131–139. https://doi.org/10.1017/S146625230999017X.

Fulton, R. W., K. S. Blood, R. J. Panciera, M. E. Payton, J. F. Ridpath, A. W. Confer, J. T. Saliki, L. T. Burge, R. D. Welsh, B. J. Johnson, and A. Reck. 2009. Lung pathology and infectious agents in fatal feedlot pneumonias and relationship with mortality, disease onset, and treatments. J. Vet. Diagn. Invest. 21:464–477. https://doi.org/10.1177/104063870902100407.

Gagea, M. I., K. G. Bateman, R. A. Shanahan, T. van Dreumel, B. J. McEwen, S. Carman, M. Archambault, and J. L. Caswell. 2006. Naturally occurring *Mycoplasma bovis*-associated pneumonia and polyarthritis in feedlot beef calves. J. Vet. Diagn. Invest. 18:29–40. https://doi.org/10.1177/104063870601800105.

Gänheim, C., A. Johansson, P. Ohagen, and K. Persson Waller. 2005. Changes in peripheral blood leucocyte counts and subpopulations after experimental infection with BVDV and/or *Mannheimia haemolytica*. J. Vet. Med. B Infect. Dis. Vet. Public Health 52:380–385. https://doi.org/10.1111/j.1439-0450.2005.00882.x.

Gersheim, L. J. 2007. Bovine respiratory syncytial virus infection: Immunopathogenic mechanisms. Anim. Health Res. Rev. 8:207–213. https://doi.org/10.1017/S1466252307001405.

Griffin, D., M. M. Chengappa, J. Kuszak, and D. S. McVey. 2010. Bacterial pathogens of the bovine respiratory disease complex. Vet. Clin. North Am. Food Anim. Pract. 26:381–394. https://doi.org/10.1016/j.cvfa.2010.04.004.

Guo, T., X. Qi, L. Zhang, X. Y. Zhao, X. J. Jia, J. Z. Xu, and C. L. Fan. 2010. Pathological study of bovine disease of *Arcanobacterium pyogenes*. Heilongjiang Anim. Sci. Vet. Med. 1:127–128. https://doi.org/10.13881/j.cnki.hljxmsy.2010.01.008.

Guo, T., J. Zhang, X. Chen, X. Wei, C. Wu, Q. Cui, and Y. Hao. 2021. Investigation of viral pathogens in cattle with bovine respiratory disease complex in Inner Mongolia, China. Microb. Pathog. 153:104594. https://doi.org/10.1016/j.micpath.2020.104594.

Holman, D. B., T. McAllister, E. Topp, A. D. G. Wright, and T. W. Alexander. 2015. The nasopharyngeal microbiota of feedlot cattle that develop bovine respiratory disease. Vet. Microbiol. 180:90–95. https://doi.org/10.1016/j.vetmic.2015.07.031.

Hou, P., G. Zhao, H. Wang, and H. He. 2019. Prevalence of bovine viral diarrhea virus in dairy cattle herds in eastern China. Trop.
