



Evaluation of bovine Parainfluenza type-3 virus and Influenza virus D participation in bovine respiratory disease of calves from Brazilian family farming

[Avaliação da participação do Parainfluenza vírus do tipo 3 e do Influenza vírus D na doença respiratória dos bovinos de bezerros de agricultura familiar]

"Artigo Científico/Scientific Article"

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Abstract

Bovine respiratory disease (BRD) is responsible for great economic losses in bovine production with major impact on family farming. Among all possible causes of bovine respiratory disease, viruses are commonly categorized as primary etiological agents. The present study aimed to identify the presence of bovine parainfluenza type 3 virus and Influenza virus D (IVD) in tracheobronchial fluid samples of healthy calves and calves showing clinical signs of bovine respiratory disease. Samples were collected from 42 family farming farms in São Paulo State, Brazil. A total of 141 tracheobronchial fluid samples from healthy calves (n=100) and calves showing clinical signs of bovine respiratory disease (n=41) were enrolled in this study. No sample was positive for both viruses after Real-Time PCR. Serum neutralization for bovine Parainfluenza type 3 virus (bPI-3v) was performed using 88 randomly selected serum samples and antibodies were detected in 45.45% (40/88) samples. Serum reactive samples were detected in both healthy (46.8%; 22/47) and BRD calves (43.9%; 18/41). Regarding antibody titer, numerical differences were detected between healthy (3%) and BRD calves (9%) for titers 128-512 ($P > 0.05$). The present research was the first that aimed to study the presence of bPI-3V and IVD in family farming herds from São Paulo State, Brazil. This research revealed that bPI-3v is circulating in the region studied and is possibly involved in some BRD cases. On the other hand, IVD was not detected.

Keywords: viruses; pneumonia; bovine production; Real-time PCR; tracheobronchial fluid samples.

Resumo

A broncopneumonia é responsável por importantes perdas econômicas na produção bovina com maior impacto em pequenas produções, como a agricultura familiar. Dentre todas as possíveis causas da pneumonia bovina, os vírus são comumente categorizados como agentes etiológicos primários. O presente estudo teve como objetivo identificar a presença de Parainfluenza bovina tipo -3 (bPI-3V) e Influenzavírus D (IVD) por meio da PCR em Tempo Real em amostras de lavado traqueobrônquico de bezerros leiteiros saudáveis e com problemas respiratórios criados nos assentamentos do Estado de São Paulo, Brasil. As amostras foram colhidas em 42 pequenas propriedades de agricultura familiar. Ao todo, 141 amostras de lavado traqueobrônquico foram coletadas de bezerros sadios (n=100) e com problemas respiratórios (n=41). Nenhuma amostra foi positiva para nenhum dos vírus após PCR em Tempo Real. Soro neutralização para bPI-3v foi realizada em 88 amostras selecionadas aleatoriamente e anticorpos foram detectados em 45,45% (40/88) das amostras. Amostras reagentes foram detectadas em ambos bezerros sadios (46,8%; 22/47) e bezerros com doença respiratória (43,9%; 18/41). Em relação ao título de anticorpos, diferenças foram detectadas entre sadios (3%) e doentes (9%) para título entre 128-512 ($P > 0.05$). A presente pesquisa foi a primeira que avaliou a presença

de bPI-3V e IVD em assentamentos do Estado de São Paulo, Brasil. Nosso estudo revelou que bPI-3V está circulando nos rebanhos daquela região, podendo estar associado a alguns casos de doença respiratória bovina. Não houve detecção de IVD nos rebanhos estudados.

Palavras-chaves: vírus; pneumonia; produção bovina; PCR em tempo real; lavado traqueobrônquico.

Introduction

Bovine respiratory disease (BRD) is responsible for high economic losses in dairy and beef cattle production (USDA, 2013), with major impact on family farming herds. Viruses are commonly categorized as primary etiological agents of BRD by changing respiratory mucosa, producing cytokines and impairing functioning of immune system cells (Bosch et al., 2013).

Bovine Parainfluenza type 3 virus (bPI-3v) belongs to order Mononegavirales and family *Paramyxoviridae* (ICTV, 2014). It is genetically and antigenically related to Human Parainfluenza type 3 virus (Murphy et al., 1999). Cattle infected by bPI-3v shows from asymptomatic to severe pneumonia symptoms. Allan et al. (1978) referred to dry cough as the first clinical sign observed in outbreaks, besides increasing on rectal temperature, inappetence, mucopurulent nasal discharge (Ellis, 2010), tachypnea, and tearing. Antibodies against bPI-3v have been detected in several countries, such as Canada (Durham and Hassard, 1990), Nigeria (Ib et al., 2005), Mexico (Solís-Calderón et al., 2007), Belgium (Pardon et al., 2011), Iran (Kojuri et al., 2011; Ezzi et al., 2013), Caribbean (Twari et al., 2016), Syria (Giangaspero et al., 1997), and Brazil (Candeias and Ribeiro, 1968; Sardi et al., 2010).

Recently, a virus with 50% homology with Human Influenza C virus was isolated from pigs (Hause et al., 2013), and bovines (Collin et al., 2015), and, after phylogenetic and serological analysis, it was classified as Influenza virus D (IVD). This new virus has already been detected in Italian (Chiapponi et al., 2016), French (Ducatez et al., 2015), and American (Hause et al., 2013) herds. Mitra et al. (2016) detected a tendency in association between IVD and bovine respiratory disease. To the best of our knowledge, there are no studies that aimed to detect the presence of IVD in Brazilian dairy herds.

Besides few studies that only evaluated the presence bPI-3v, there are no researches that aimed to detect bPI-3v and IVD in family farming herd. Therefore, the present study aimed to identify the presence of bovine parainfluenza type 3 virus and Influenza virus D in tracheobronchial

fluid samples of healthy calves and calves showing clinical signs of bovine respiratory disease breeding in family farming herds.

Materials and Methods

Area definition and sampling

The State of São Paulo is located in the southeast region of Brazil (21°49'S; 49°12'W) and it has a dairy cattle population of 1,287,509 (IBGE, 2014). Pontal do Paranapanema is located in the extreme west region of the State of São Paulo. The present study was carried out in three municipalities: Caiuá (21°49'55''S; 51°59'52''O), Presidente Epitácio (21°45'53''S; 52°06'19''O) and Mirante de Paranapanema (22°17'31''S; 51°54'21''O). One-hundred and forty-one male and female, one to 12 months age, mongrel dairy calves were enrolled in this study. They received colostrum and milk from their mothers. After weaning, calves received pasture and mineral salt ad libitum.

Case definition and sample collection

Calves were randomly selected in each farm. Physical examination was performed in order to classify them as healthy calves and BRD calves. We considered BRD calves those that showed at least two of the following parameters: mucopurulent or purulent nasal discharge, cough, crackle, snoring, respiratory rate above 40 breaths per minute and rectal temperature above 39.5°C (Benesi et al., 2013).

Tracheobronchial fluid samples were collected after trichotomy and antisepsis of the trachea. An Intracath® (BD, New Jersey, USA) was introduced by tracheocentesis, and 20 mL of sterile saline 0.9% were instilled, recovering up to 5mL. A fraction was added to a dry cryogenic tube and immediately stored in nitrogen. Eight milliliters of total blood were obtained by jugular vein puncture using a dry BD vacutainer® tube. All tubes were centrifuged at 600 x g for 15 minutes and the serum obtained was stored in tubes at -20°C until further analysis.

Viral RNA was extracted from tracheobronchial fluid samples using MagMAX™ Viral RNA Isolation (Thermo Fisher Scientific Inc., Waltham, MA), according to manufacturer's instructions. For the bPI-3v and IDV detection, primers and probes used were designed by Horwood et al., (2008) and Horwood and Mahony (2011). All reactions were performed in a total volume of 15 µL containing 3.5 µL of Buffer; 0.25 µL enzyme (25X RT-PCR Enzyme - Ambion); 1.0 µL probe; 1.0 µL of each primer (50 pmol), 5.25 µL sterile deionized water and 5µL of RNA. Reactions were performed using OneStep StepOne™ Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific Inc., Waltham, MA), with the amplification cycle establish by Horwood and Mahony (2011). Results above the automatic threshold were considered positive. To ensure that samples were viable, samples were kept in nitrogen until laboratory analysis. Besides, the elution buffer used during the RNA extraction had an RNA preservative.

Serum-neutralization for bPI-3v

Serological tests were performed according to the Code of Federal Regulations (CFR, 2005). Briefly, the quantification of antibodies started from 1:2 to 1:256 in 96-well plates. Then, 100 TCID₅₀/50mL were added to bPI-3v (ATCC VR 281). All plates were incubated at 37° C with 5% CO₂ for one hour. After that, bovine kidney epithelial cell (MDBK) suspension was added and plates were incubated again in same conditions for 72 hours. Antibody titers were expressed as the inverse of the dilution where absence of cytopathic effect was observed. Samples with

titers equal or higher than three were considered reactive. Serum neutralization tests were validated through the control of negative and positive sera for anti-BVDV antibodies, control of cells, and retitration plate, according to Reed e Muench (1938).

Statistical analysis

Descriptive results were provided. All results were analyzed using Chi-square test and Fisher's Exact Test and Odds Ratio using JMP Pro 12 (SAS Institute Inc., NC). A confidence interval of 90% was used. Results showing $P < 0.01$ were considered significant.

Results

Samples were collected between 2014 and 2015 from 42 small farms located in Caiuá, Presidente Epitácio, and Mirante do Paranapanema. Hundred and forty-one calves were enrolled in this study and classified as healthy (n=100) and BRD calves (n=41). No sample was positive for either viruses by Real-Time PCR (Data not shown). Serum-neutralization for bPI-3v was performed using 88 randomly selected serum samples (47 healthy and 41 BRD calves). Antibodies were detected in 45.45% (40/88) samples. Serum reactive samples were detected in both healthy calves (46.80%; 22/47) and BRD calves (43.90%; 18/41). Regarding antibody titer, numerical differences were detected between healthy (3%) and BRD calves (9%) for titers 128 to 512 ($P > 0.05$). On the other hand, antibody titers between 2 and 8 were numerically higher in healthy (22%) calves compared to BRD calves (17%) (Figure 1).

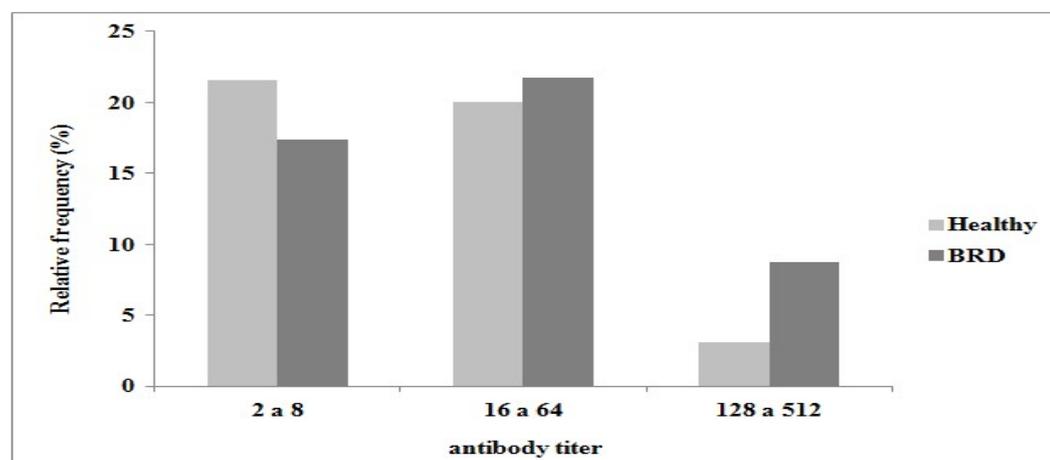


Figure 1. Antibody titers against bPI-3v detected in healthy and BRD calves by serum neutralization.

Discussion

Respiratory diseases are characterized as a multi-factorial condition (Griffin et al., 2010) that contributes to the failure of the calf breeding (Autio et al., 2007). Intracellular viral replication is responsible for cellular lysis (Bosch et al., 2013) and disorders in immune cells functionality. In an effort to detect the presence of bPI-3v and IVD in settlements from Pontal do Paranapanema, São Paulo State, Brazil, Real-Time PCR was performed using tracheobronchial fluid samples of healthy and BRD calves, besides serum neutralization tests.

In the present study, bPI-3v and IVD were not detected in tracheobronchial fluid samples of both healthy and BRD calves. The detection of the antigen in Brazilian cattle was only described in the States of São Paulo (SP) and Rio Grande do Sul (RS). Candeias and Ribeiro (1971) reported the first isolation of bPI-3v in Brazil from pulmonary tissue with pneumonic lesions of bovines in SP. On the other hand, Gonçalves et al. (2003) isolated bPI-3v from nasal discharge of a bovine with respiratory disease in RS, indicating that bPI-3v can be detected in both upper and lower respiratory tract.

Serum neutralization was performed for bPI-3v in samples randomly selected and antibodies were detected in 45.45% samples. The first indication of the presence of bPI-3v in Brazil was reported in 1968 by Candeias and Ribeiro (1968), in which study 36.71% of cattle showed antibodies against bPI-3v by inhibition of hemagglutination. Using same serological test, Sardi et al. (2010) detected antibodies in 68.5% samples in the State of Bahia. Our results demonstrate that bPI-3v is present in herds from Pontal do Paranapanema and it addresses the need for constant monitoring. It is well known that bPI-3v is an important primary agent of BRD, although this virus predisposes to secondary bacterial infection (Ellis, 2010). The detection of antigen is possible when the animal has an active infection. The presence of antibodies against bPI-3v in addition to the failure to detect the antigen could be related to the possibility of animals show a regressive infection and have developed specific immune responses (Griffin et al. 2010).

To the best of our knowledge, this is the first study that aimed to detect Influenza virus D in Brazilian herds by molecular approaches. The presence of IVD was already reported in the

United States (Hause et al., 2013, Ferguson et al., 2015), Italy (Chiapponi et al., 2016), and France (Ducatez et al., 2015). There is no evidence that this virus is circulating in South America, especially in those countries bordering Brazil. To the authors' knowledge, no antibody test for IVD is available. Considering IVD as a new species, more information is required to better understand its pathology, cross-reactivity to other influenza viruses and epidemiology.

Conclusion

The present research was the first that evaluated the presence of bPI-3v and IVD in family farming farms located in São Paulo State, Brazil. Our research revealed that bPI-3v seems to be important to pneumonia cases in that place. Moreover, studies should be expanded to better understand the importance of bPI-3v and to confirm the absence of IVD in Brazilian family farming dairy herds.

Conflict of Interest

The authors have no competing interests.

Ethics statement

The present study was conducted at the Laboratory of Clinical and Molecular Virology from Institute of Biomedical Sciences, Laboratory of Bovine Virology from the Biologic Institute, at the School of Veterinary Medicine and Animal Science, University of São Paulo, Brazil, from August 2014 until March 2016. All procedures were carried out in agreement with the guidelines Committee of Ethics on Animal Use (Protocol number: 7973040214).

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