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Detection of Respiratory Viral Pathogens and *Mycoplasma* spp from Calves with Summer Pneumonia in Korea

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Abstract : Respiratory pathogens of calves including bovine parainfluenza type 3 virus (BPI3V), bovine respiratory syncytial virus (BRSV), infectious bovine rhinotracheitis virus (IBRV) and *Mycoplasma* spp is well-known for winter pathogens. However, there are no studies about summer pneumonia pathogens of calves in Korea. The aim of this study was to detect respiratory pathogens from calves with summer pneumonia. Eighty calves from 5 regions were chosen and their nasal swabs were used to detect respiratory pathogens with real-time PCR. *Mycoplasma* spp was major primary respiratory pathogens in calves with summer pneumonia. Although, the detection rates of respiratory viruses were very low, serological assays showed that respiratory viruses exist widely in farms.

Key words **:** respiratory viral pathogens, *Mycoplasma*, calves, real-time PCR.

Introduction

Respiratory disease of calves is a principal disease problem for the cattle industry which causes great economic losses. In economic terms, respiratory disease of calves leads to declined production, higher levels of mortality and morbidity, increased veterinary costs, and reduced carcass value (3,10,21). While the etiological factors including stress and environmental factors such as temperature, humidity, livestock density, dust, transportation, and inadequate nutrition are various, infectious agents are important in the development of disease (5,6,19,21).

Frequently, bacterial pneumonia is preceded by a viral respiratory infection. Respiratory viruses, which are effectively spread by aerosol and direct contact in herds, usually act in combination with other infectious agents, in particular bacteria, in the development of respiratory disease (4,19). The primary respiratory pathogens are bovine parainfluenza type 3 virus (BPI3V), bovine respiratory syncytial virus (BRSV), infectious bovine rhinotracheitis virus (IBRV), coronavirus, adenovirus, and *Mycoplasma* spp (2,11,14,17,20). BPI3V replicates in epithelial cells of lower respiratory tract causing bronchitis, bronchiolitis, alveolitis and destruction of cilia and of ciliated cells in bronchi and bronchioli. BPI3V infects alveolar macrophages and thereby impairs innate pulmonary defense mechanisms (3,15,20). BRSV infection results in destruction of the ciliated respiratory epithelium and infection of alveolar macrophages reduces local cellular immunity. This interference with pulmonary clearance predisposes cattle to secondary bacterial pulmonary infection (2,20,24). IBRV, which is bovine herpesvirus 1, replicates in mucosal cells and in various cell types of the submucosa and connective tissue peripheral to the tracheal rings. This may lead to destruction of the epithelium of the upper respiratory tract with cessation of ciliary activity resulting in loss of function of the mucociliary escalator. Consequently, secondary bronchopneumonia may occur due to inhalation of infectious tracheal exudates (20). Furthermore, on farms where bovine viral diarrhea virus (BVDV) is not well controlled, this can result immune-suppression and influence the progression of respiratory disorders (7,18). *Mycoplasma* spp is common inhabitant of the upper and lower respiratory tract of healthy and pneumonic cattle. The lesions of pneumonia associated with *Mycoplasma* spp infection are characterized by subacute or chronic bronchopneumonia with multiple foci of caseous and coagulative necrosis (14). Infection with respiratory viruses and *Mycoplasma* spp can also induce opportunistic secondary pathogens such as *Mannheimia haemolytica*, *Pasteurella multocida*, *Haemophilus somni*, etc (1,8,13,17).

The peak incidence of clinical respiratory diseases of calves usually occurs in late autumn and winter in Korea. More rapid and severe temperature changes and greater weather extremes would contribute to higher morbidity and mortality rates of respiratory diseases. However, there is no study about summer pneumonia pathogens of calves in Korea. The aim of this study was to detect primary respiratory viral pathogens and *Mycoplasma* spp from calves which have summer pneumonia in Korea.

Materials and Methods

Clinical samples

One hundred and twenty five calves (2 weeks to 5 months after birth) with clinical signs of respiratory disease were selected from 5 regions (Chuncheon, Paju, Seosan, Pyeongchang, and Andong). Total respiratory score was measured

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according to the calf respiratory scoring criteria (CRSC) from the University of Wisconsin (16) and eighty calves with total score of 4 and above were sampled by deep nasal swab (1).

Extraction of viral RNA and bacterial DNA

QIAamp viral RNA kit (QIAGEN, Hilden, Germany) was used to extract viral RNA and IBRV DNA. QIAamp DNA kit (QIAGEN) was used to extract *Mycoplasma* DNA.

Reverse transcription

cDNAs were amplified by real-time PCR machine (LightCycler 96, Roche Life Science, Basel, Switzerland). The reverse transcription was carried with PrimeScript RTase kit (TaKara Bio, California, USA) according to the included protocol. The 20 μl volume containing 4 μl of 5X RT buffer [50 mM Tris-HCl (pH 8.3), 75 mM KCl, 8 mM $MgCl_2$, 10 mM DTT], 1 μl of a 10 mM dNTP mix, 1 μl of random primer (500 μg/ml), 0.5 μl of PrimeScript reverse transcriptase (200 U/μl), 0.5 μl of RNase inhibitor RNAguard (40 U/μl), and 3μl DEPC-treated water to 10 μl of viral RNA. The conditions of synthesizing cDNA were 10 min at 30°C, 30 min at 50°C, and 15 min at 75°C.

Primers

The primers of BPI3V, BRSV, IBRV, BVDV, and *Mycoplasma* used in this study are shown in Table 1 (22,25,26).

Real-time PCR assay

The Power SYBR Green PCR Master Mix with real-time PCR system was used. The PCR conditions were as followed: For LightCycler reaction, a mastermix of the following reaction components was prepared to indicated end-concentration: 10 μl of Fast Start DNA Master SYBR Green I, 4.5 μl distilled water, 4 μl primer (1 pmol). LightCycler mastermix was filled in the LightCycler glass capillaries and 1.5 μl cDNA was added as PCR template. Capillaries were closed, centrifuged and placed into the LightCycler rotor. The following LightCycler experimental run protocol was used: preincubation program (95°C for 10 min), 3 step amplification program repeated 45 cycles (95°C for 10 s, 60°C for 10 s, and 72°C for 10 s), melting curve program (95°C for 10 s, 65°C for 60 s, and 97°C for 1 s). Detection of respiratory pathogens from calves was regarded when mean Cq values were under 35.

Virus-neutralizing antibody

Blood was collected from jugular vein to test virus-neutralizing antibody against BPI3V, BRSV, and IBRV. The 50 μl of α-minimal essential medium (α-MEM) with 200 $TCID_{50}$ on each virus was added to 50 μl of heat-inactivated two-fold diluted sera already prepared in 96 wells plates. And then, plate was incubated at 37°C for one hour. A total of 100 μl of the MDBK cell (for BPI3V and IBRV) or bovine turbinate cell (for BRSV) suspension (2×10^5 cells/ml) added to each well, and the plate was incubated at 37°C in 5% CO_2 for 5-6 days. Finally, it was screened for the presence or absence of cytopathic effects using phase-contrast optics and inverted microscope. Seropositive was regarded when serum neutralizing antibody titer against BPI3V, BRSV, and IBRV were 4 and above.

Results

The detection sensitivity of real-time PCR assay for BPI3V, BRSV, and IBRV included in INFORCE 3 vaccine (Zoetis, USA) was determined by 10-fold serial dilutions ($10^{5.5}$ $TCID_{50}$ to $10^{0.5}$ $TCID_{50}$ of PI3V, $10^{5.2}$ $TCID_{50}$ to $10^{1.2}$ $TCID_{50}$ of BRSV, and $10^{6.0}$ $TCID_{50}$ to $10^{2.0}$ $TCID_{50}$ of IBRV, Fig 1). The results show positive log linear correlations of viral copy number and PCR quantitative cycle (Cq) number. The standard curves show accuracy of $R^2 > 0.98$.

Among 80 tested samples, analysis by real-time PCR detected one strain (1.3%) of IBRV, three strains (3.8%) of BVDV, sixty one strains (76.3%) of *Mycoplasma* spp (Table 2).

Among 80 test sera, 30 sera (37.5%) were seropositive against BPI3V, 34 (42.5%) against BRSV, and 32 (40.0%) against IBRV (Table 3).

Table 1. Primers of real-time PCR for BPI3V, BRSV, IBRV, BVDV, and *Mycoplasm*a

Pathogens	Primers*	Sequences (5′-3′)
BPI3	BPI3euro Forward	GGTAGGAGCACCTCCACGATT
	BPI3euro Reverse	GCTCCAAGGCATGCTGGATA
BRSV	BRSVn Forward	GGTCAAACTAAATGACACTTTCAACAAG
	BRSVn Reverse	AGCATACCACACAACTTATTGAGATG
IBRV	gB Forward	TGTGGACCTAAACCTCACGGT
	gB Reverse	GTAGTCGAGCAGACCCGTGTC
BVDV	BV7	ACTCCATGTGCCATGTACAGC
	BV8	TAGCCATGCCCTTAGTAGGAC
Mycoplasma	GPO-3	GGGAGCAAACA GGATTAGATACCCT
	MGSO	TGCACCATCTGTCACTCT GTTAACCTC
β-Actin	Bac1F_uni	GACAGGATGCAGAARGAGATCAC
	Bac2R_uni	TCCACATCTGCTGGAAGGTG

*The primer of BPI3V is based on conserved regions of euro gene of BPI3V, the primer of BRSV is based on conserved regions of N gene of BRSV, the primer of IBRV is based on conserved regions of glycoprotein B gene of bovine herpesvirus-1, the primer of BVDV is based on conserved regions of the 5'UTR of BVDV genotype I and II and the *Mycoplasma* group-specific primer is based on highly conserved 16S rRNA genes.

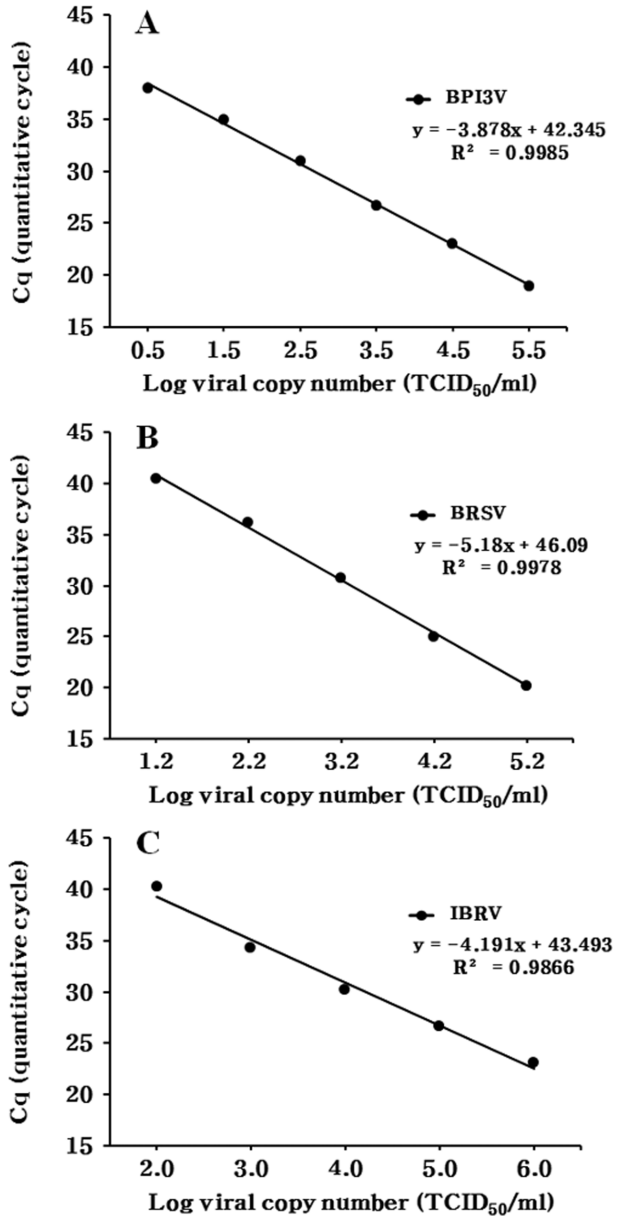

Fig 1. The standard curves of the real-time PCR assay for the detection of BPI3V (A), BRSV (B), and IBRV (C).

Table 2. Detection of respiratory pathogens from calves with summer pneumonia by real-time PCR

Pathogens	Tested samples	No. of positive*	Positive rates (%)
BPI3V	80	0	0
BRSV	80	0	0
IBRV	80	1	1.3
BVDV	80	3	3.8
Mycoplasma spp	80	61	76.3

*Detection of respiratory pathogens from calves was regarded when mean Cq values were under 35.

Discussion

The availability of rapid, sensitive and cost effective diagnostics are important for effective management of livestock health. Such results are also important from an epidemiological perspective to provide an accurate picture of the prevalence and distribution of pathogens in different cattle populations and production systems, with such information being fundamental to the development of appropriate management and vaccination strategies. In terms of direct detection of viral pathogens, molecular techniques have displaced previous assays, such as virus isolation and immunofluorescent antibody staining due to significant advantages in speed, sensitivity, sample lability and cost (22,27). The recent development and application of test methods based on the PCR for the direct detection of respiratory pathogens, particularly when applied on real-time platforms, represents a significant step forward in the investigation of BRD outbreaks. These assays offer rapid, sensitive and cost-effective diagnostics which, alongside the updating of sampling protocols, offer improved detection rates and greater confidence in laboratory results. And in this study, a β-actin signal was detected in all clinical samples tested in real-time PCR, suggesting no evidence of extraction failure or PCR inhibition (22,27).

Table 3. Seropositivity of calves with summer pneumonia against BPI3V, BRSV, and IBRV

Virus	Tested samples	No. of seropositive*	Positive rates (%)
BPI3V	80	30	37.5
BRSV	80	34	42.5
IBRV	80	32	40.0

*Seropositive was regarded when serum neutralizing antibody titer against BPI3V, BRSV, and IBRV were 4 and above.

BRD in calves is a function of the interaction between pathogens, host and environment. Many different pathogens may be involved with viruses considered to be the most important in calves (9,23). BRD is a respiratory syndrome linked to presence of infectious agents, but also to management, environment, stress and immunity factors. A wide range of pathogens, essentially viruses and bacteria, can cause BRD, separately or simultaneously (12,16). Housing quality plays a particularly important role in the development of respiratory disease in young calves, alongside other factors, such as prevailing climate, biosecurity and nutrition of the calf and dam (16).

In this study, we only tested BPI3V, BRSV, IBRV, BVDV and *Mycoplasma* spp as respiratory pathogens by real-time PCR and *Mycoplasma* spp was the most frequently found respiratory pathogen. The role of *Mycoplasma* spp are mainly associated with subclinical infections and are suspected to play a role in the induction of BRD by negatively affecting the immune system and upper respiratory defense mechanism. *Mycoplasma* spp identified by PCR in lung tissue have been reported to be associated with pneumonic lesions in young dairy calves (1,8,11,17). *Mycoplasma* spp also associated with chronic debilitating diseases that react poorly to treatment (8).

In this study, BPI3V and BRSV were not detected from calves with summer pneumonia. In addition, the detection rates of IBRV and BVDV were very low. However, the seroprevalences of BPI3V, BRSV, and IBRV were 37.5%, 42.5%,

and 40.0%, respectively. Although viruses implicated in BRD appear to be maintained within herds year-round, there may be distinct seasonal trends (9,12,16). Irish study found that positive detections of the various viruses are strongly influenced by the month of sampling, with distinct intermonth variation as to when detections are most likely. However, with the exception of BVDV, which was detected all year round, the others showed a clear seasonal pattern, being most commonly detected in winter and spring (16). BRD mainly occurred during the colder months, suggesting the influence of conditions like low temperatures, high humidity, and high population densities (9).

Furthermore, viral secretions in the nasal cavity are generally of a short duration (usually under 1 week, depending on the virus), which makes viral detection difficult if repetitive sampling is not conducted (24). Consequently, it is embarrassing to conclude on the basis of the low detection rates of these viruses that they are not significant pathogens in calves with summer pneumonia. The herds in this study are representative of the open housing, commercially driven dairy and beef enterprises in Korea, systems which may differ from those seen in other countries. In addition to clinical presentation, history and other factors, strategic herd health planning should take into account the time of year to best prioritize the most likely respiratory pathogens to prepare against. A pronounced seasonality suggests that optimum times may exist for active mitigation, so in the future, specific diseases alerts could become a meaningful industry tool.

We have done additional experimentation with 60 deep nasal samples conducting in autumn from calves which have respiratory disease. Analysis by real-time PCR detected one strain (1.7%) of PI3V and seventeen (28.3%) of IBRV (Data not shown). As getting cold, virus detection rates improved, and IBRV was more prevalent than other viruses. These viral infections of calves commonly occur worldwide, and can cause serious respiratory diseases mainly in autumn and winter (16). Although viral infections seem rare during summer, these virus-induced diseases and infections annually recur in a herd, even when the herd is closed and virus reintroduction is doubtful. A previous study showed that asymptomatic infections took place in spring and summer, and the results also suggested that viruses are more likely to persist in individual seropositive cattle than by continuous circulation (20). It may be seemed that such persistent viral infections are activated again in autumn or winter and act as the cause of a new outbreak. This is because viruses are better transmitted during winter. Climatic differences, especially getting cold, might make the proper viruses in the environment viable and permit virus shedding in different periods. Gradually warmer temperatures should slowly reduce the spreading of viruses during the summer. Cooling of the nasal mucosa is thought to increase the viscosity of the mucous layer and reduce the frequency of cilia beats that could help clear viruses. In this way, breathing cold and dry air would impair mucociliary clearance, inhibit leukocyte phagocytosis and thereby promote viral shedding within the respiratory tract. Improved persistence of released virus would increase the amount of shedding viruses, and would furthermore boost proliferation of virus in the nasal passages through reinfection (6). In short, respiratory viral detection rates of calves with summer pneumonia in Korea were low, however, it might rise from winter.

Although BVDV was detected in some of the samples tested in this study, its pattern of detection appears distinct from those of the more classical respiratory viruses. Infections occur at a lower and much steadier rate, with less seasonal variation. The result from this study would suggest a lower prevalence linking BRD and BVDV as a primary pathogen in calves compared to some previous studies (12,16).

In conclusion, real-time PCR method is capable of detection of respiratory pathogens of calves with summer pneumonia. This study showed that *Mycoplasma* spp was more prevalent than viruses for respiratory pathogens in calves with summer pneumonia. The detection rates of primary respiratory viruses were very low in calves with summer pneumonia. However, serological assays showed that respiratory viruses are also spread widely in farms.

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References

1. Allen JW, Viel L, Bateman KG, Rosendal S, Shewen PE, Physick-Sheard P. The microbial flora of the respiratory tract in feedlot calves: associations between nasopharyngeal and bronchoalveolar lavage cultures. Can J Vet Res 1991; 55: 341-346.
2. Baker JC, Frey ML. Bovine respiratory syncytial virus. Vet Clin North Am Large Anim Pract 1985; 1: 259-275.
3. Bryson DG. Calf pneumonia. Vet Clin North Am Large Anim Pract 1985; 1: 237-257.
4. Callan RJ, Garry FB. Biosecurity and bovine respiratory disease. Vet Clin Food Anim 2002; 18: 57-77.
5. Cusack PM, McMeniman N, Lean IJ. The medicine and epidemiology of bovine respiratory disease in feedlots. Aust Vet J 2003; 81: 480-487.
6. Eccles R. An explanation for the seasonality of acute upper respiratory tract viral infections. Acta Otolaryngol 2002; 122: 183-191.
7. Ellis JA. The immunology of the bovine respiratory disease complex. Vet Clin North Am Food Anim Pract 2001; 17: 535-550.
8. Francoz D, Buczinski S, Belanger AM, Forte G, Labrecque O, Tremblay D, Wellemans V, Dubuc J. Respiratory pathogens in Québec dairy calves and their relationship with clinical status, lung consolidation, and average daily gain. J Vet Inter Med 2015; 29: 381-387.
9. Gay E, Barnouin J. A nation-wide epidemiological study of acute bovine respiratory disease in France. Prev Vet Med 2009; 89: 265-271.
10. Griffin D. Economic impact associated with respiratory disease in beef cattle. Vet Clin North Am Food Anim Pract 1997; 13: 367-377.
11. Gulliksen SM, Jor E, Lie KI, Løken T, Akerstedt J, Østerås O. Respiratory infections in Norwegian dairy calves. J Dairy Sci 2009; 92: 5139-5146.
12. Hagglund S, Svensson C, Emanuelson U, Valarcher JF, Alenius

S. Dynamics of virus infections involved in the bovine respiratory disease complex in Swedish dairy herds. Vet J 2006; 172: 320-328.
13. Hotchkiss EJ, Dagleish MP, Willoughby K, McKendrick IJ, Finlayson J, Zadoks RN, Newsome E, Brulisauer F, Gunn GJ, Hodgson JC. Prevalence of *Pasteurella multocida* and other respiratory pathogens in the nasal tract of Scottish calves. Vet Rec 2010; 167: 555-560.
14. Khodakaram-Tafti A, Lopez A. Immunohistopathological findings in the lungs of calves naturally infected with *Mycoplasma bovis*. J Vet Med 2004; 51: 10-14.
15. Maidana SS, Lomonaco PM, Combessies G, Craig MI, Diodati J, Rodriguez D, Parreno V, Zabal O, Konrad JL, Crudelli G, Mauroy A, Thiry E, Sonia A, Romera SA. Isolation and characterization of bovine parainfluenza virus type 3 from water buffaloes (*Bubalus bualis*) in Argentina. BMC Vet Res 2012; 8: 83. doi: 10.1186/1746-6148-8-83.
16. O'Neill R, Mooney J, Connaghan E, Furphy C, Graham DA. Patterns of detection of respiratory viruses in nasal swabs from calves in Ireland: a retrospective study. Vet Rec 2014; 175: 351. doi: 10.1136/vr.102574.
17. Pardon B, De Bleecker K, Dewulf J, Callens J, Boyen F, Catry B, Deprez P. Prevalence of respiratory pathogens in diseased, non-vaccinated, routinely medicated veal calves. Vet Rec 2011; 169: 278. doi: 10.1136/vr.d4406.
18. Pellerin C, van den Hurk J, Lecomte J, Tijssen P. Identification of a new group of bovine viral diarrhea virus strains associated with severe outbreaks and high mortalities. Virology 1994; 203: 26-268.
19. Sloan C, Moore ML, Hartert T. Impact of pollution, climate, and sociodemographic factors on spatiotemporal dynamics of seasonal respiratory viruses. Clin Trans Sci 2011; 4: 48-54.
20. Smith BP. The bronchopneumonias (respiratory disease complex of cattle, sheep, and goats). In: Large Animal Internal Medicine. 4th ed. St Louis, Missouri: Mosby Elsevier. 2009: 602-613.
21. Snowder GD, Van Vleck LD, Cundiff LV, Bennett GL. Bovine respiratory disease in feedlot cattle: environmental, genetic, and economic fact. J Anim Sci 2006; 84: 1999-2008.
22. Thonur L, Maley M, Gilray J, Crook T, Laming E, Turnbull D, Nath M, Willoughby K. One-step multiplex real time RT-PCR for the detection of bovine respiratory syncytial virus, bovine herpesvirus 1 and bovine parainfluenza virus 3. BMC Vet Res 2012; 8: 37-45.
23. Van der Fels-Klerx HJ, Horst HS, Dijkhuizen AA. Risk factors for bovine respiratory disease in dairy youngstock in the Netherlands: the perception of experts. Livest Prod Sci 2000; 66: 35-46.
24. Van der Poel WH, Kramps JA, Middel WG, Van Oirschot JT, Brand A. Dynamics of bovine respiratory syncytial virus; a longitudinal epidemiological study in dairy herds. Arch Virol 1993; 133: 309-321.
25. Van Kuppeveld FJ, van der Logt JT, Angulo AF, van Zoest MJ, Quint WG, Niesters HG, Galama JM, Melchers WJ. Genus- and species-specific identification of mycoplasmas by 16S rRNA amplification. Appl Environ Microbiol 1992; 58: 2606-2615.
26. Wang J, O'Keefe J, Orr D, Loth L, Banks M, Wakeley P, West D, Card R, Ibata G, Van Maanen K, Thoren P, Isaksson M, Kerkhofs P. Validation of a real-time PCR assay for the detection of bovine herpesvirus 1 in bovine semen. J Virol Methods 2007; 144: 103-108.
27. Willoughby K, Thomson K, Maley M, Gilray J, Scholes S, Howie F, Caldow G, Nettleton P. Development of a real time reverse transcriptase polymerase chain reaction for the detection of bovine respiratory syncytial virus in clinical samples and its comparison with immuno-histochemistry and immuno-fluorescence antibody testing. Vet Microbiol 2008; 126: 264-270.