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Fatemeh Roshtkhari, Gholamreza Mohammadi & Ashraf Mayameei

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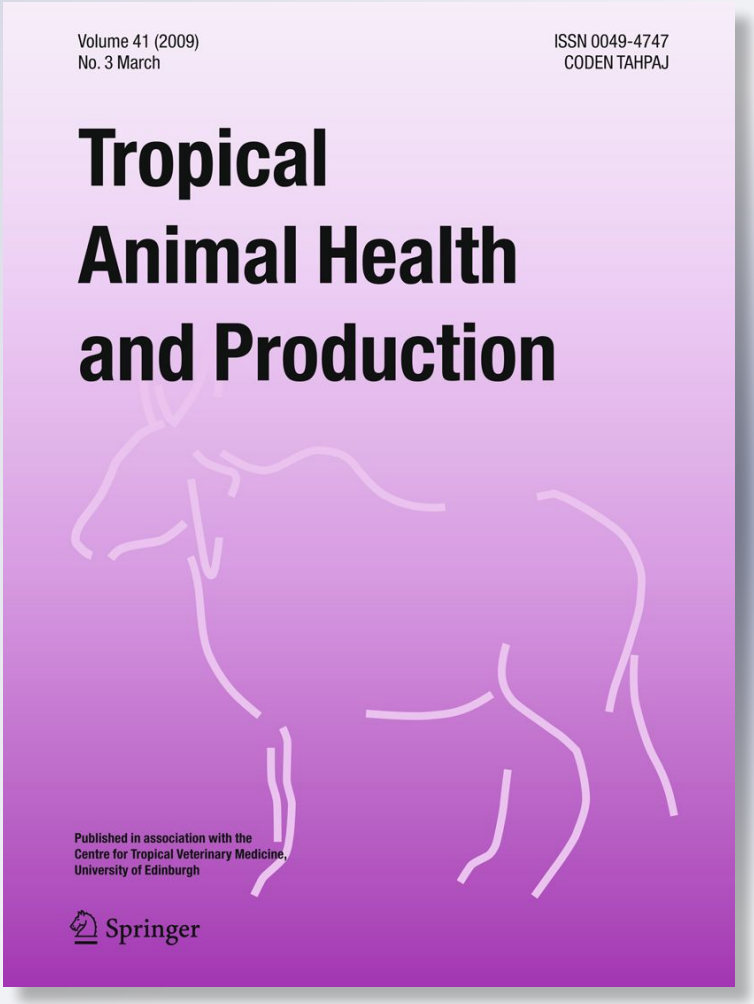

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ORIGINAL RESEARCH

Serological evaluation of relationship between viral pathogens (BHV-1, BVDV, BRSV, PI-3V, and Adeno3) and dairy calf pneumonia by indirect ELISA

Fatemeh Roshtkhari · Gholamreza Mohammadi · Ashraf Mayameei

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Abstract In this study, viral pathogens associated with nine outbreaks of naturally occurring dairy calf pneumonia in Mashhad area of Khorasan Razavi province from September 2008 to May 2009 were assessed. Five diseased calves from each farm were chosen for examination. Acute and convalescent serum samples were taken from calves with signs of respiratory disease. Sera were analyzed for antibodies to bovine viral diarrhea virus (BVDV), bovine herpesvirus type 1 (BHV-1), bovine respiratory syncytial virus (BRSV), parainfluenza virus type 3 (PI-3V), and bovine adenovirus-3 (BAV-3) by indirect ELISA kits. Among 42 serum samples collected at sample 1, seroprevalence values for viruses BHV-1, BVDV, BRSV, PI-3V, and BAV-3 were 61.9% (26), 57.1% (24), 64.2% (27), 90% (38), and 61.9% (26), respectively. Seroconversion to BVDV, BRSV, PI-3V, and BAV-3 occurred in 11.9% (5), 16.6% (7), 26.1% (11), and 21.4% (9) of animals, and 52.3% (22) had generated antibodies against one or more viral infections at sample 2. In addition, no significant relationship between seroprevalence of BHV-1, BVDV, BRSV, PI-3V, and BAV-3 and dairy herd size was observed ($P>0.05$). According to serological findings, BHV-1, BVDV, BRSV, PI-3V, and BAV-3 are common pathogens of the dairy calf pneumonia in dairy herds in Mashhad area of Khorasan Razavi province, Iran.

Keywords Dairy calf pneumonia · BHV-1 · BVDV · BRSV · PI-3V · BAV-3 · Indirect ELISA

Abbreviations

BAV	Bovine adenovirus
BAV-3	Bovine adenovirus-3
BCV	Bovine coronavirus
BHV-1	Bovine herpesvirus -1
BRD	Bovine respiratory disease
BRSV	Bovine respiratory syncytial virus
BVDV	Bovine viral diarrhea virus
DCP	Dairy calf pneumonia
ELISA	Enzyme-linked immunosorbent assay
PI-3V	Bovine parainfluenza virus 3

Introduction

Respiratory diseases in calves are responsible for major economic losses in both the beef and dairy production industries (Van der Fels-Klerx et al. 2001; Thomson and White 2006). Alone or in association with other pathogens, viral infections are probably the main causes of these diseases. It is generally accepted that viruses are the first pathogens to intervene, whereas bacteria act as secondary invaders which worsen the already-ill animal's condition (Valarcher and Hägglund 2006; Solis Calderon et al. 2007; Taylor et al. 2010). The most important viruses in cattle are bovine respiratory syncytial virus (BRSV), bovine parainfluenza virus 3 (PI-3V), bovine viral diarrhea virus (BVDV), bovine coronavirus (BCV), bovine adenovirus (BAV), and

F. Roshtkhari
School of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran

G. Mohammadi (✉)
Department of Clinical Sciences, School of Veterinary Medicine, Ferdowsi University of Mashhad,
P.O. Box: 91775-1793, Mashhad, Iran
e-mail: gmohamad@um.ac.ir

A. Mayameei
Department of Pathobiology, School of Veterinary Medicine, Ferdowsi University of Mashhad,
P.O. Box: 91775-1793, Mashhad, Iran

bovine herpesvirus-1 (BHV-1), the causative agent of infectious bovine rhinotracheitis (IBR). Four of these viral pathogens, BVDV, BHV-1, BRSV, and PI-3V are mainly associated with bovine respiratory disease (Ellis 2009; Brodersen 2010; Schneider et al. 2010). These agents cause severe disruption of the respiratory tract and are associated with shipping fever in weaned and growing cattle as calves are transported to feedlots for finishing. Moreover, BHV-1 and BVDV can suppress the immune system of the host and increase the risk of secondary bacterial infections and/or mycoplasma-associated respiratory diseases (Valarcher and Hägglund 2006; Jones and Chowdhury 2010). BVDV is one of the most important pathogens in cattle worldwide (Houe 1995). Serosurveys of cattle from different countries indicate that the prevalence of antibodies to BVDV may range from 40% to 90% (Houe 1995; Kirkland 1996).

IBR is caused by BHV-1 and is considered to have a worldwide distribution. BHV-1 can become latent in the trigeminal ganglia and tonsils. Latency allows the virus to persist, so that the introduction of a carrier into a non-infected herd is the principle way for the spread of the virus. For these latent infections, positive serology means the animal is a potential carrier of the virus (Winkler et al. 2000). Outbreaks of respiratory disease involving BRSV have been reported in many places in Europe, the USA, and other parts of the world (Ohlson et al. 2010). PI-3V infections cause less serious disease than BRSV (Valarcher and Hägglund 2006) but are nevertheless significantly correlated with respiratory diseases in cattle (Kadir and Burak 2008). Several surveys given in many countries have revealed that infection with PI-3V is endemic in both beef and dairy cattle (Obando et al. 1999). This paper reports a study of nine outbreaks of dairy calf pneumonia and attempts to investigate the possible involvement of BVDV, BHV-1, BRSV, PI-3V, and BAV-3 in outbreaks of dairy calf pneumonia (DCP) in nine dairy herds in Mashhad area of Khorasan Razavi province, Iran, by using indirect ELISA.

Materials and methods

Animals and herds

The entire study was carried out in Mashhad area, capital city of the Khorasan Razavi province, from September 2008 to May 2009. Mashhad has an estimated 70,000 cattle and more than 500 dairy farms. The herd size varied from farm to farm (with an average size of 50 milking cows per herd) with the most common cattle breed being Holstein/Friesian. Nine outbreaks of dairy calf pneumonia were investigated in nine separate dairy herds. The farms were selected based on the outbreaks of DCP and the operators' willingness to cooperate (Table 1). The cow breed on all nine farms was Holstein/Friesian. None of the dairy herds in this study were vaccinated against BVDV, BHV-1, BRSV, PI-3V, or BAV-3. Herds were separated into two groups. The size of the dairy herds in group 1 (four out of nine herds) varied between 283 and 571 cows per herd (mean, 427), and the size of the dairy herds in group 2 (five out of nine herds) had 1,229–1,937 cows per herd (mean, 1,583). Five diseased calves from each farm were chosen for closer, clinical examinations (such as evaluation of lung sound with a stethoscope and vital signs); altogether, 45 calves were examined. It was necessary to ascertain whether or not the unvaccinated animals had already seroconverted (had been exposed to natural infection) (Thrusfield 2007). Each calf was examined; birth date was recorded, and age in days was calculated. The age of the diseased calves varied from 80 to 140 days (mean, 110). A respiratory disease score was assigned based on rectal temperature, the character of nasal discharge, eye or ear appearance, and presence of a cough based on the method by Lago et al. (2005). The respiratory disease score is the sum of points from the four categories of clinical signs, with increasing values representing progressive severity. The scoring system resulted

Table 1 Seropositivity rate of viruses detected based on serum samples from 42 calves in 9 dairy herds in Mashhad area of Khorasan Razavi province, Iran

Group	Herds	Number of calves	Seropositivity rates (%), sample 1					Seropositivity rates (%), sample 2				
			BHV-1	BVDV	BRSV	PI-3V	BAV-3	BHV-1	BVDV	BRSV	PI-3V	BAV-3
1	A	5	80	40	100	80	60	80	60	60	100	100
	B	5	100	80	100	100	60	60	60	40	80	60
	C	5	0	40	60	100	60	0	0	100	100	100
	D	4	75	100	0	75	25	75	75	0	0	50
2	E	5	40	100	20	100	100	60	100	40	100	100
	F	5	80	0	100	80	60	80	20	100	80	20
	G	5	80	100	60	100	100	40	100	100	100	40
	H	5	40	40	80	80	20	20	80	100	100	100
	I	3	66.6	0	33.3	100	66.6	0	0	100	100	100

in a minimum score of 0 and a maximum score of 12. Calves with a score of 6 or higher had at least two clinical signs of respiratory disease and were thus considered sick (Table 2). Three calves died 2–5 days after the examination and were necropsied.

Blood sample collection

Blood samples for serological studies were taken from all calves as early as possible in the course of infection/disease (acute sample or sample 1), and second samples were taken from 42 calves 3 to 4 weeks later (convalescent sample or sample 2). Blood samples (5 ml) were collected aseptically from the jugular vein of each animal using anticoagulant free Vacutainer tubes and transported on ice to the virology laboratory of the School of Veterinary Medicine, Ferdowsi University of Mashhad, on the same day. Serum was separated by centrifugation at 3,000×*g* for 10 min at room temperature; aliquots were transferred into 1.5-ml sterile microtubes, and tubes were stored at −20°C until analysis.

Detection of viral antibodies

Commercial indirect respiratory ELISA kit (Pentakit) developed by Bio-X Diagnostics, Belgium, was used to determine the presence of antibodies to BVDV, BHV-1, BRSV, PI-3V, and BAV-3. Ninety-six-well microtiter plates were coated by monoclonal antibodies specific to the five pathogens listed above. The ELISA procedure was performed according to the manufacturer's instruction. The optical density (OD) was measured at 450 nm with an ELISA plate reader (BioTek Instruments, USA). Calculating the net OD of each sample and determining each serum's degree of positivity were performed according to manufacturer instruction. A sample was considered positive if it yielded a result that was greater than or equal to one plus sign (+). A frank seroconversion was considered to have occurred if the signal increased by two orders of magnitude (for example, ++>++++ or +>+++).

Statistical analysis

Statistical significance of differences in seroprevalence values between two groups of the herds was analyzed using chi-square analysis and Fischer's exact test where appropriate (SPSS software version-16). Statistical significance was considered obtained when *P* values were less than 0 .05.

Results

The serological status of sampled calves is shown in Table 3. In sample 1, PI-3V showed the highest seroprevalence when compared to BRSV, BAV-3, BHV-1, and BVDV. In sample 2, BRSV, BAV-3, and BVDV exhibited increased seroprevalence, whereas PI-3V and BHV-1 demonstrated decreased seroprevalence.

According to the classification described above, 74% of the animals were infected with three or more monitored viruses in sample 1, and these percentages rose to 88% in sample 2 (Table 4). Seroconversion to BVDV, BRSV, PI-3V, and BAV-3 occurred in 11.9% (5), 16.6% (7), 26.1% (11), and 21.4% (9) of animals between the first and second serum sampling, respectively. Seroconversion for BHV-1 was not noted in any of the calves. Overall seroconversion occurred in 22 of 42 calves (52.3%) on nine farms.

Of all animals, 65.4% to 89.5% of seropositive animals in sample 1, from which paired sera were observed, remained seropositive in sample 2, BHV-1, 17 out of 26 (65.4%); BVDV, 20 out of 24 (83.3%); BRSV, 22 out of 27 (81.5%); PI-3V, 34 out of 38 (89.5%); BAV-3, 20 out of 26 (76.9%). In agreement with the individual serological data for the nine farms, seroconversion for PI-3V, BAV-3, BRSV, and BVDV was seen on four, five, four, and three of the farms, respectively (Table 5). In addition, no significant differences were observed between the seroprevalence of BHV-1, BVDV, BRSV, PI-3V, and BAV-3 with respect to dairy herd size ($P>0.05$).

Table 2 Scoring system for calf respiratory disease (Lago et al. 2005)

Clinical sign	Points allocated for signs below			
	0	1	2	3
Rectal temperature (°C)	37.8–38.2	38.3–38.8	38.9–39.3	≥39.4
Cough	None	Induce single	Induce repeated or occasional spontaneous cough	Repeated spontaneous coughing
Nasal discharge	Normal serous	Small amount of unilateral, cloudy	Bilateral, cloudy, or excessive mucus	Copious bilateral, mucopurulent nasal discharge
Eye or ear	Normal	Mild ocular discharge	Bilateral purulent ocular discharge or unilateral ear drop	Head tilt or both ears dropped

Table 3 Seroprevalence for BHV-1, BVDV, BRSV, PI-3V, and BAV-3 of calves with dairy calf pneumonia in nine dairy herds in Mashhad area of Khorasan Razavi province, Iran

Virus	Sample 1, positive animals[a] (%, *N*=42)	Sample 2, positive animals[a] (%, *N*=42)	Negative to positive[b] (%, *N*=42)	Positive to negative[c] (%, *N*=42)
BHV-1	61.9 (26)	47.6 (20)	7.1 (3)	21.4 (9)
BVDV	57.1 (24)	61.9 (26)	9.5 (4)	9.5 (4)
BRSV	64.2 (27)	71.4 (30)	19 (8)	12 (5)
PI-3V	90 (38)	85.7 (36)	4.7 (2)	9.5 (4)
BAV-3	61.9 (26)	73.8 (31)	26.1 (11)	14.2 (6)

[a] Number of seropositive animals detected in samples 1 and 2, respectively

[b] Number of animals that seroconverted between samples 1 and 2

[c] Number of animals that converted from a positive sample 1 to a negative in sample 2

Discussion

Viral infections of the bovine respiratory tract represent significant pathogens. These infections are manifested by various clinical signs and lesions in DCP with varying degrees of morbidity, mortality, loss of production (treatment costs, reduced weight gain and carcass value), and lowered economic return to the producer. A wide range of infectious agents have been implicated in DCP. The principal viruses include BRSV, PI-3V, BAV, and BCV; other more common viruses, such as BVDV and BHV-1, are rarely involved in DCP unless a farm problem involving these viruses in the adult cattle is occurring (Fulton 2009).

Viruses in DCP may cause primary infection with disease, either singly or in combination with other viruses. A significant role for viruses in DCP is their interaction with bacteria and *Mycoplasma* spp. in bacterial pneumonias (Fulton et al. 2000).

Young calves are especially susceptible to viral infections because their maternally derived immunity from colostrum is reduced with age (Saif 2004). Calves held under stressful conditions such as markets, commingling during marketing and shipment, inadequate nutrition, overcrowding, and severe climatic changes are more prone to DCP. Often calves fresh from closed herds of the ranch operation are highly susceptible as they enter the marketing channels and are commingled with other calves, facilitating the spread of the viruses. Viruses are shed primarily in respiratory secretions of the nose, eyes, and sometimes feces. Direct or close contacts with animals' infectious secretions are major modes of transmission. The morbidity rate and mortality rate (case fatality) are often low but can be much higher depending on bacterial agents such as *Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni*, or *Mycoplasma* spp. (*Mycoplasma bovis*) (Valarcher and Hägglund 2006).

The diagnosis of specific etiologic agents requires the use of diagnostic laboratory tests. Diagnosis of an active infection with field strains of virus can be made by detecting changes in antibody titers in acute to convalescent serum samples. An acute sample should be collected as early as possible in the course of infection/disease and the convalescent sample 3 to 4 weeks later. The results of the present study demonstrate that PI-3V, BRVS, BVDV, BHV-I, and BAV-3 are commonly associated with DCP in dairy herds in Mashhad area of Khorasan Razavi province, Iran. Additionally, a recent study shows that concomitant multiple viral infections are encountered in outbreaks of DCP in dairy herds as indicated by concomitant seroconversion to more different viral agents in diseased animals. Serological evidence of one to five different

Table 4 Number of animals infected with combinations of 0–5 viruses (BHV-1, BVDV, BRSV, PI-3V, and BAV-3), based on paired serum samples from 42 calves in 9 dairy herds in Mashhad area of Khorasan Razavi province, Iran (five calves per herd)

Number of infections[a]	Sample 1 (%)	Sample 2 (%)
5	8 (19)	5 (11.9)
4	12 (28.5)	14 (33.3)
3	11 (26.1)	18 (42.8)
2	9 (21.4)	3 (7.1)
1	2 (4.7)	0
0	0	2 (4.7)

[a] Any combination of BHV-1, BVDV, BRSV, PIV-3, and BAV-3; for definition of infected animal

Table 5 Number of herds classified as infected with BHV-1, BVDV, BRSV, PIV-3, and BAV-3 and based on paired serum samples from 42 calves in 9 dairy herds in Mashhad area of Khorasan Razavi province, Iran (five calves per herd)

Viruses	Sample 1[a] (%)	Sample 2[a] (%)
BHV-1	8 (88.8)	7 (77.7)
BVDV	7 (77.7)	7 (77.7)
BRSV	8 (88.8)	8 (88.8)
PI-3V	9 (100)	8 (88.8)
BAV-3	9 (100)	9 (100)

[a] For classification of infection

viral agents in a herd during a DCP was observed, and infection with two or more different viruses in one diseased animal with the observed 32 rising antibodies to seroconversion viruses (BVDV, BRSV, PI-3V, and BAV-3) was proved in 22 diseased calves. This situation may be characterized by the multi-agent nature of the etiology of DCP (Autio et al. 2007). Multiple antibody detections in an animal may be due to the contribution of these viruses in a BRD case (Hagglund et al. 2006); concurrently, those antibodies might also be generated by individual virus infections caused at different time intervals. Although we detected antibodies to several viruses on each farm and even in high titers, we could only find seroconversion in 22 of 42 diseased calves on nine farms. This is probably because of the young age of the calves and existing maternal antibodies which may suppress the calves' own production of antibodies. This is in agreement with the results of a high number of decreasing antibody titers and with the conclusion of Virtala et al. (1996), who stated that young pneumonic calves often fail to seroconvert to agents present in the respiratory tract because of suppressive maternal antibodies. Our failure to detect seroconversion might also be a consequence of missing the acute phase of the disease.

This study shows that herd size is not a risk factor for BHV-1, BVDV, BRSV, PI-3V, and BAV-3 infections. It is probably due to the similar intensive management system in both groups. Therefore, it is suggested that the main problem is the spreading of viruses by adult cattle in those herds (Yeşilbağ and Güngör 2008). The animal populations on all farms were separated, with no movement of animals into or out of each herd. Separated animal populations are less likely to be infected from other areas than are contiguous populations. However, if infection enters separated populations, it may spread rapidly because the animal density frequently is high. Consequently, in this situation, adult cattle are the reservoirs of viral infection—serving as the source of exposure to susceptible calves in nine herds. Infected animals can be identified relatively easily by using laboratory diagnostics (such as PCR) and can be removed by slaughter or quarantine (Thrusfield 2007). Viral infections can be also reduced in these herds by increased biosecurity aimed at reducing the risk of potential transmission of infection.

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