Detection and quantification of parapoxvirus DNA by use of a quantitative real-time polymerase chain reaction assay in calves without clinical signs of parapoxvirus infection

Gakuji Yaegashi DVM

Kazuhiro Fukunari DVM

Takayuki Oyama DVM

Ryu-koh Murakami DVM

Yasuo Inoshima DVM, PhD

Received March 31, 2015.
Accepted June 22, 2015.

From Iwate Prefecture Central Livestock Hygiene Service Center, 390-5 Sunakomi, Takizawa, Iwate 020-0605, Japan (Yaegashi, Fukunari, Oyama, Murakami); and the Cooperative Department of Veterinary Medicine, Faculty of Applied Biological Sciences, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan (Inoshima).

Address correspondence to Dr. Inoshima (inoshima@gifu-u.ac.jp).

OBJECTIVE
To investigate the presence of parapoxvirus (PPV) in cattle without clinical signs of infection and in farm environments of PPV-infected cattle.

ANIMALS
28 calves without clinical signs of PPV infection on 2 farms and 11 clinically affected calves on 6 farms.

PROCEDURES
164 oral swab samples were collected at regular intervals from 28 calves without clinical signs of PPV infection, and 11 swab samples were collected from 11 clinically affected calves. Viral DNA load was quantified by use of a PPV-specific quantitative real-time PCR (qRT-PCR) assay.

RESULTS
Of 28 calves without clinical signs of PPV infection, 12 had positive results for PPV DNA by use of the qRT-PCR assay. Viral DNA was detected continuously over a period of 2 to 5 months from 9 of these 12 calves, particularly from calves with dermatomycosis or respiratory tract disease. The PPV DNA loads in 32 oral swab samples from these 12 calves were significantly lower (median, 3.2 copies/mg) than those in samples collected from the 11 clinically affected calves (median, 3.2×10^4 copies/mg). Moreover, PPV DNA was detected in the residual feed and drinking water on both farms that housed the calves without clinical signs of PPV infection.

CONCLUSIONS AND CLINICAL RELEVANCE
PPV in cattle without clinical signs of infection and in the environments of these cattle may represent sources of PPV transmission to susceptible cattle.

IMPACT FOR HUMAN MEDICINE
Humans should wear gloves to prevent zoonotic disease transmission when handling cattle with or without clinical signs of PPV infection. (*Am J Vet Res* 2016;77:383–387)

The genus *Parapoxvirus* in the family Poxviridae comprises 4 members: orf virus, BPSV, pseudocowpox virus, and parapoxvirus of red deer in New Zealand.[1] Parapoxvirus infections are widespread in ruminants throughout the world and are generally characterized by mild papules and erosions on the muzzle, oral mucosa, and udder. However, mucosal disease, infectious bovine rhinotracheitis, malignant catarrhal fever, and foot-and-mouth disease cause similar lesions in affected cattle.[2,3] It is known that PPV can be transmitted to humans by close contact with affected animals or PPV-contaminated materials. The disease in humans is a zoonotic occupational disease for farmers, veterinarians, and abattoir workers.[4–8]

ABBREVIATIONS

BPSV	Bovine papular stomatitis virus
PPV	Parapoxvirus
qRT-PCR	Quantitative real-time PCR
RFLP	Restriction fragment length polymorphism

Parapoxvirus infection is typically diagnosed on the basis of clinical signs, isolation of the virus, and detection of viral antigen or virus particles in lesions during histologic and electron microscopic examinations.[9–12] The PCR assays are recognized as fast and sensitive diagnostic methods and have been used widely to detect viruses.[13–16] Real-time PCR assays are used routinely for detection of various viral pathogens, whereas real-time amplification techniques are used to quantify viral load in clinical samples.[17] A real-time PCR method for detection of PPV has been reported.[18–20]

Parapoxvirus often induces subclinical infections in herds. There may be numerous subclinically infected animals, and it has been suggested these subclinically affected animals act as a reservoir of infection.[21–24] Furthermore, it has been reported that 31 of 45 (68.9%) swab samples obtained from the oral cavity of calves at a livestock market in the United States had positive results for PPV DNA, as determined by use of a real-time PCR assay.[25] Although a quantitative PCR assay for estimating PPV viral load has been de-

scribed for clinically affected cattle,[18–20] practical approaches for determining viral load in cattle subclinically infected with PPV remain poorly defined. There is a paucity of information on viral load in cattle subclinically infected with PPV.

We believed that cattle infected with PPV but that did not have clinical signs of PPV infection might be a source for infections on farms. The study reported here was conducted to determine the virologic status of cattle subclinically infected with PPV and the farm environment of those cattle. We compared the PPV DNA load of calves without clinical signs of PPV infection with the DNA load of calves clinically affected with PPV.

Materials and Methods

Animals

A total of 39 calves were examined. During routine medical examination of cattle (foot-and-mouth disease surveillance) performed by veterinarians, 28 calves without clinical signs of PPV infection and with no history of PPV infection were randomly selected from 2 farms in Iwate Prefecture, Japan (24 Holstein calves from farm 1 and 4 Japanese Black calves from farm 2). Calves were between 9 days and 16 months of age during the investigation. Samples were collected continuously between September 2011 and March 2012.

Samples were obtained from 11 calves with clinical signs of PPV infection from 6 farms and used for comparison. Calves had lesions such as papules, erosions, and ulcers in the mucosa of the lips, gingiva, or tongue. None of the calves had lesions on teats or in the interdigital cleft of limbs. Calves were between 2 and 9 months of age. Samples were collected once from each calf between April 2010 and September 2013.

The study was approved by the Gifu University Animal Care and Use Committee. Owner consent was obtained for use of animals in the study and collection of samples from the calves and farm environment.

Sample collection

Oral swab samples (n = 164) were collected by use of cotton-tipped swabs[a] from calves without clinical signs of PPV infection. Each sample weighed approximately 200 mg. Samples were suspended in 2 mL of PBS solution. Suspensions were centrifuged at 2,500 × *g* for 10 minutes, and the supernatant was harvested and used for qRT-PCR assay. Similarly, oral swab samples (n = 11) were collected from mucosal lesions of 11 PPV-affected calves.

Samples were obtained to assess the farm environment for calves without clinical signs of PPV infection. Approximately 200 mg of residual feed and 200 µL of drinking water were collected 7 times from farm 1 and 5 times from farm 2.

Virus isolation

Swab-sample suspensions obtained from calves with clinical signs of PPV infection were passed through a 0.45-µm filter[b] and then inoculated onto primary bovine fetal testis cells and bovine fetal muscle cells; cells were incubated at 37°C for 1 hour. Cells were washed twice with Eagle minimum essential medium[c] and then were cultured at 37°C in Eagle minimum essential medium containing 5% fetal calf serum until cytopathic effects were observed. When cytopathic effects were not observed after culture for 14 days, cells were freeze-thawed 3 times and inoculated onto fresh bovine fetal testis cells or bovine fetal muscle cells, which were again cultured until cytopathic effects were observed; the freeze-thaw process was repeated twice, if necessary. Swab-sample suspensions obtained from calves without clinical signs of PPV infection were not used for virus isolation.

qRT- PCR assay

Total DNA was extracted from swab samples with a commercial kit[d] used in accordance with the manufacturer's instructions. A qRT-PCR assay was performed as reported elsewhere.[19] An aliquot (5 µL) of template DNA was added to 12.5 µL of *Taq*,[e] 7.5 pmol of each primer (PPV forward, TCGATGCGGTGCAGCAC; PPV reverse, GCGGCGTATTCTTCTCGGAC), 2.5 pmol of minor groove binder probe (FAM-TGCGGTAGAAGCC-NFQ), 1µM reference dye,[f] and distilled water (final volume of reaction mixture, 25 µL). The PCR amplification was performed by use of a real-time PCR system.[g] Thermal cycling conditions were 5 minutes at 95°C and 40 cycles of denaturation at 95°C for 15 seconds and annealing and extension at 60°C for 32 seconds. Standard DNA for the qRT-PCR assay was prepared from DNA of BPSV strain IW2010A (GenBank accession No. AB920996)[26] by use of a PCR assay with pan-parapoxvirus primers-1 and -4, as described elsewhere.[13] The PCR amplicon was purified by use of a commercial kit,[h] adjusted to 1.0×10^8 copies/µL, and quantified by use of a spectrophotometer.[i] Serial dilutions of standard DNA ranging from 0.5×10^6 copies/reaction to 5×10^6 copies/reaction were tested to detect clinical samples. Mean viral load was determined from duplicate measurements. Detection limits were 5×10^{-2} copies/reaction for a 10-fold dilution of the standard DNA, as determined in a preliminary experiment.

The DNA samples in which PPV DNA was detected by use of the qRT-PCR assay were subjected to RFLP analysis to classify the PPV species, as described elsewhere.[27] The RFLP analysis was performed by use of the restriction enzymes *Drd* I, *Xmn* I, *Pfl*M I, and *Hin*c II.

Statistical analysis

The PPV DNA loads obtained from calves with and without clinical signs of PPV infection were analyzed by use of the Mann-Whitney *U* test. Values were considered significant at $P < 0.01$.

Results

Calves without clinical signs of PPV infection

Of the 28 calves without clinical signs of PPV infection, PPV DNA was detected by use of the qRT-PCR assay in samples obtained from 8 of 24 (33.3%) calves from farm 1 and 4 of 4 calves from farm 2. In addition, BPSV was identified on the basis of results for a PCR assay and RFLP analysis in samples obtained from these 12 calves (data not shown). Age of the 12 calves with positive results for the qRT-PCR assay ranged from 9 days to 13 months. The PPV DNA was detected only once from 3 of the 12 calves, whereas PPV DNA was detected numerous times over a period of 2 to 5 months from the other 9 calves. During the latter part of the study, PPV DNA was not detected in samples obtained from calves on farm 1.

Among the 9 calves with numerous samples that yielded PPV DNA, 1 calf from farm 1 had severe dermatomycosis, and 4 calves from farm 2 had respiratory tract disease. The calf with dermatomycosis had numerous gray-white, rough, and hairless lesions on the body extending from the head (ear, muzzle, and periorbital skin) to the tail. Respiratory tract disease was characterized by mucoid nasal discharge, coughing, rapid breathing, and fever in the 4 affected calves. Parapoxvirus DNA was detected continuously from those 5 calves between 3 and 5 months.

The PPV DNA load was measured in 32 oral swab samples obtained from 16 calves apparently not infected with PPV on the 2 farms. The PPV DNA load ranged from 6.1×10^{-2} copies/mg to 5.7×10^{2} copies/mg (median, 3.2 copies/mg; **Figure 1**).

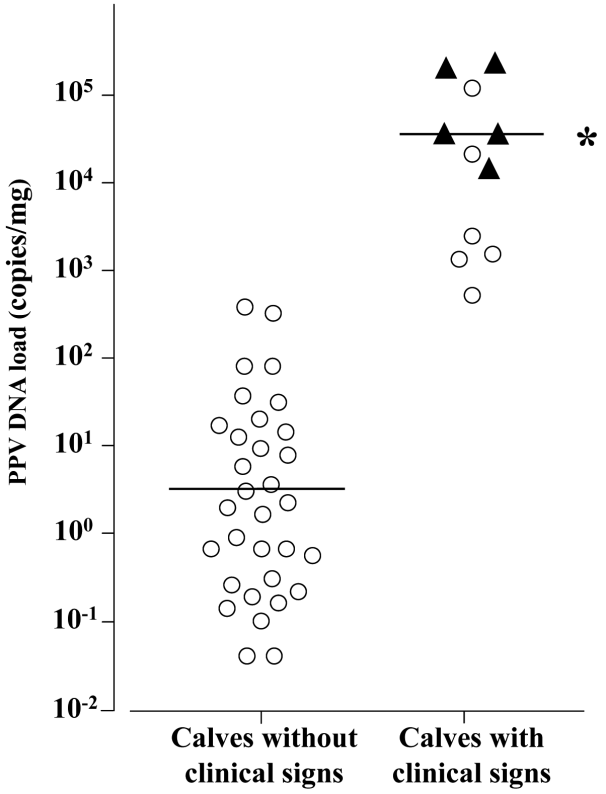

Figure 1—Comparison of PPV DNA load in swab samples obtained from calves with or without clinical signs of PPV infection. Age of the 32 calves without clinical signs of PPV infection (2 farms) and the 11 calves with clinical signs of PPV infection (6 farms) ranged from 9 days to 13 months and from 2 to 9 months, respectively. Each symbol represents results for 1 calf; samples from which PPV was isolated are indicated (triangles). Median values for each group are indicated (horizontal lines). *Median value differs significantly ($P < 0.001$) from the median value for the other group of calves.

Calves with clinical signs of PPV infection

Multiple papular lesions were observed on the lips, gingiva, and tongues of 11 calves with clinical signs of PPV infection. Lesions on the lips typically were found in calves < 3 months old **(Table 1)**. The PPV DNA was detected in the 11 swab samples obtained from the 11 affected calves. In addition, BPSV was identified on the basis of results for a PCR assay and RFLP analysis in samples obtained from all 11 calves (data not shown). The PPV DNA load in the samples ranged from 7.8×10^{2} copies/mg to 3.4×10^{5} copies/mg (median, 3.2×10^{4} copies/mg). The PPV DNA load of each affected calf was higher than that for calves apparently not infected with PPV (Figure 1). Cells inoculated with swab suspensions obtained from 5 calves with clinical signs of PPV infection had cytopathic effects in bovine fetal testis cells or bovine fetal muscle cells (or both) after the second passage.

Typically, virus isolation was more successful for samples obtained from calves with relatively high PPV

Table 1—Relationship between location of lesions and age of calf versus lesion status for detection of PPV DNA in swab samples obtained from 11 calves with clinical signs of PPV infection.

	Papular lesion			Erosion			Healing lesion		
Location	+	–	*P* value*	+	–	*P* value*	+	–	*P* value
Lip†	3.0 (n = 7)	5.5 (n = 4)	0.005	3.0 (n = 6)	5.0 (n = 5)	0.017	3.0 (n = 5)	4.5 (n = 6)	0.037
Gingiva†	3.0 (n = 6)	5.0 (n = 5)	0.320	3.0 (n = 6)	3.0 (n = 5)	1.000	4.0 (n = 1)	3.0 (n = 10)	ND
Tongue†	9.0 (n = 1)	3.0 (n = 10)	ND	5.5 (n = 2) ‡	3.0 (n = 9)	ND	5.0 (n = 1)	3.0 (n = 10)	ND

Results for a qRT-PCR assay were positive (+) or negative (–). Values in in parentheses represent numbers of calves with positive and negative assay results. Overall, 9 calves had papular lesions, 9 had erosions, and 7 had healing lesions.

*Values were considered significant at $P < 0.01$. †Value reported is the median age of calves from which samples were obtained; calves ranged from 2 to 9 months of age. ‡Value reported is the mean.

ND = Not determined.

Table 2—Detection of PPV DNA in samples of residual feed and drinking water obtained from 2 farms with calves that had no clinical signs of PPV infection.

		2011								2012			
Farm	Source	Aug 11	Sep 22	Sep 26	Oct 13	Oct 24	Nov 7	Nov 29	Nov 30	Jan 30	Feb 3	Mar 14	Mar 22
1	Residual feed	+	–	NT	+	NT	+	NT	+	NT	+	–	NT
	Drinking water	–	+	NT	–	NT	–	NT	–	NT	–	–	NT
2	Residual feed	NT	NT	+	NT	+	NT	+	NT	+	NT	NT	+
	Drinking water	NT	NT	–	NT	–	NT	+	NT	+	NT	NT	+

Results for a qRT-PCR assay were positive (+) or negative (–).
NT = Not tested.

DNA loads. Virus was isolated at higher viral DNA loads (2.0×10^4 copies/mg to 3.4×10^5 copies/mg) but not at lower viral DNA loads (7.8×10^2 copies/mg to 1.9×10^5 copies/mg; Figure 1). A significant ($P < 0.001$) difference in PPV DNA load was detected between the calves without clinical signs of PPV infection (12 calves; 32 swab samples) and the calves with clinical signs of PPV infection (11 calves; 11 swab samples).

Detection of PPV in environmental samples

Parapoxvirus DNA was detected in residual feed and drinking water of both farms. Positive results were obtained by use of the qRT-PCR assay **(Table 2)**.

Discussion

The PPV viral load in calves without clinical signs of PPV infection has not previously been determined. Surprisingly, we confirmed that calves were highly likely to be subclinically infected with PPV. During collection of samples from calves of 2 farms at regular intervals, viral DNA was detected in 12 of 28 (42.9%) calves without clinical signs of PPV infection. These 12 calves were between 9 days and 13 months of age. Detection of PPV in 3 calves was transient (detected only once), whereas PPV DNA was continuously detected for 2 to 5 months in the other 9 calves. However, viral DNA load was significantly lower for the 12 calves without clinical signs of PPV infection than for the 11 calves with clinical signs of PPV infection. Investigators of a study[25] in the United States reported that 31 of 45 (68.9%) swab samples obtained from calves without clinical signs of PPV infection at a livestock market had positive results when tested for PPV DNA. Although the rates for positive results differed, conceivably because of differences in sample collection, number of locations (2 farms vs 1 livestock market), virologic characteristics of circulating strains, and country of the study, the results reported here suggested that apparently uninfected cattle harbor and transmit PPV continuously.

Although clinical signs of PPV infection have been reported in adult cattle,[28] clinical signs are generally seen in young cattle,[29,30] which is consistent with the fact that calves with and without clinical signs of PPV infection in the study reported here were from 2 to 9 months of age and 9 days to 13 months of age, respectively. Moreover, most of the affected calves < 3 months old had multiple and severe lesions. Furthermore, it appeared that no clinical signs developed in infected calves with oral viral DNA loads < 5×10^2 copies/mg.

Papular lesions of BPSV infection appear on the muzzle, rostral aspect of the nares, and oral cavity. Initially, they begin as small papules, progress to epithelial hyperplasia, and then become necrotic and shallow craterous lesions surrounded by a slightly raised red margin. After the lesions form a scab, they heal.[29,31] In the present study, lesions were confirmed in various regions of affected calves from 2 to 9 months of age. Healing lesions were not observed in 4 calves, which suggested that those animals may have been in the early stages of infection. However, in these affected calves, although relatively large quantities of PPV DNA were detected, definitive relationships were not observed between the amount of PPV DNA and the presence of lesions. During virus isolation, PPV was isolated from swab samples containing > 10^4 copies/mg. On the other hand, there were a few swab samples from which PPV was not isolated during virus isolation despite relatively large viral DNA loads, which indicated no obvious relationship between viral load and virus isolation. It has been reported that PPV isolation can be unsuccessful even though viruses are detected in lesions by use of electron microscopy, immunohistochemical analysis, or PCR assay.[13,16,26] These results suggest that viral DNA load in oral swab samples may not correlate with the success of virus isolation.

The PPV DNA was continuously detected in samples obtained from 5 calves without clinical signs of PPV infection in the study reported here. This appeared to be related to severe dermatomycosis and respiratory tract disease. There were no PPV-specific signs or lesions in the 5 calves. Clinical signs of PPV infection are induced by stress factors that trigger a modification of immunoreactions.[32] Although the precise stress factors were unclear during the present study, dermatomycosis and respiratory tract disease might have influenced the immunologic conditions of some calves, which resulted in PPV activation and continuous detection of PPV DNA.

A positivity rate of 40% to 98% for antibodies against PPV has been reported for cattle in Japan.[33] This rate can be attributed to the resistant nature of virions in the environment and to the transport of cattle. In the present study, PPV DNA was detected in resid-

ual feed and drinking water on 2 farms. Therefore, it is possible that PPV shed by infected calves contaminated the farm environment and that the virus was then transmitted to other cattle via water and food. Results of the present study also suggested that the presence of cattle infected with PPV but that do not have clinical signs of PPV infection might act as an infectious source of PPV on farms. In addition, even if clinical signs are not confirmed, it is likely that viral transmission and dissemination are the result of increased viral load induced by stress factors such as dermatomycosis and respiratory tract disease. Thus, strict health and hygiene management is necessary to decrease viral infections and the risk of disease onset.

Furthermore, it is known that PPV can be transmitted to humans by close contact with affected animals or PPV-contaminated materials. Therefore, we recommend that people wear gloves to prevent viral infection when handling cattle with or without clinical signs of PPV infection.

Acknowledgments

Supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan (No. 23380178), and by the Kieikai Research Foundation.

Footnotes

a. Men-tip, Japan Cotton Buds Industry Ltd, Tokyo, Japan.
b. Minisart-plus, Sartorius Stedim Biotech, Goettingen, Germany.
c. Eagle's MEM Nissui, Nissui, Tokyo, Japan.
d. QIAamp DNA mini kit, Qiagen, Valencia, Calif.
e. Premix Ex Taq, Perfect Real Time, Takara Bio, Ōtsu, Japan.
f. Rox Reference Dye II, Perfect Real Time, Takara Bio, Ōtsu, Japan.
g. ABI 7500 real-time PCR system, Applied Biosystems, Foster City, Calif.
h. QIAquick PCR purification kit, Qiagen, Valencia, Calif.
i. GeneQuant 100, GE Healthcare, Little Chalfont, Buckinghamshire, England.

References

1. Skinner MA, Buller RM, Damon IK, et al. Family Poxviridae. In: King AMQ, Adams MJ, Carstens EB, et al, eds. *Virus taxonomy. Ninth report of the International Committee on Taxonomy of Viruses*. London: Elsevier, 2012;291–309.
2. Griesemer RA, Cole CR. Bovine papular stomatitis. *J Am Vet Med Assoc* 1960;137:404–410.
3. Mayr A, Büttner M. Bovine papular stomatitis virus. In: Dinter Z, Morein B, eds. *Virus infections of ruminants*. Vol 3. Amsterdam: Elsevier, 1990;23–28.
4. Büttner M, Rziha H-J. Parapoxviruses: from the lesion to the viral genome. *J Vet Med B Infect Dis Vet Public Health* 2002;49:7–16.
5. Damon IK. Poxviruses. In: Knipe DM, Howley PM, eds. *Fields virology*. 6th ed. Philadelphia: Lippincott Williams and Wilkins, 2013;2160–2184.
6. Friedman-Kien AE, Rowe WP, Banfield WG. Milker's nodules: isolation of a poxvirus from a human case. *Science* 1963;140:1335–1336.
7. Robinson AJ, Petersen GV. Orf virus infection of workers in the meat industry. *N Z Med J* 1983;96:81–85.
8. Schnurrenberger PR, Swango LJ, Bowman GM, et al. Bovine papular stomatitis incidence in veterinary students. *Can J Comp Med* 1980;44:239–243.
9. Becher P, König M, Müller G, et al. Characterization of sealpox virus, a separate member of the parapoxviruses. *Arch Virol* 2002;147:1133–1140.
10. Guo J, Rasmussen J, Wünschmann A, et al. Genetic characterization of orf viruses isolated from various ruminant species of a zoo. *Vet Microbiol* 2004;99:81–92.
11. Mazur C, Ferreira II, Rangel Filho FB, et al. Molecular characterization of Brazilian isolates of orf virus. *Vet Microbiol* 2000;73:253–259.
12. Torfason EG, Guðnadóttir S. Polymerase chain reaction for laboratory diagnosis of orf virus infections. *J Clin Virol* 2002;24:79–84.
13. Inoshima Y, Morooka A, Sentsui H. Detection and diagnosis of parapoxvirus by the polymerase chain reaction. *J Virol Methods* 2000;84:201–208.
14. Leonard D, Otter A, Everest D, et al. Unusual bovine papular stomatitis virus infection in a British dairy cow. *Vet Rec* 2009;164:65.
15. Oem JK, Lee EY, Lee KK, et al. Bovine papular stomatitis virus (BPSV) infections in Korean native cattle. *J Vet Med Sci* 2013;75:675–678.
16. Takahashi M, Seimiya YM, Seki Y, et al. Bovine papular stomatitis in a Japanese Black calf [in Japanese]. *Nippon Juishikai Zasshi* 2010;63:359–363.
17. Mackay IM, Arden KE, Nitsche A. Real-time PCR in virology. *Nucleic Acids Res* 2002;30:1292–1305.
18. Gallina L, Dal Pozzo F, McInnes CJ, et al. A real time PCR assay for the detection and quantification of orf virus. *J Virol Methods* 2006;134:140–145.
19. Nitsche A, Büttner M, Wilhelm S, et al. Real-time PCR detection of parapoxvirus DNA. *Clin Chem* 2006;52:316–319.
20. Zhao H, Wilkins K, Damon IK, et al. Specific qPCR assays for the detection of orf virus, pseudocowpox virus and bovine papular stomatitis virus. *J Virol Methods* 2013;194:229–234.
21. Greig A, Linklater KA, Clark WA. Persistent orf in a ram. *Vet Rec* 1984;115:149.
22. Iketani Y, Inoshima Y, Asano A, et al. Persistent parapoxvirus infection in cattle. *Microbiol Immunol* 2002;46:285–291.
23. Nettleton PF, Gilray JA, Yirrell DL, et al. Natural transmission of orf virus from clinically normal ewes to orf-naive sheep. *Vet Rec* 1996;139:364–366.
24. Snider TG III, McConnell S, Pierce KR. Increased incidence of bovine papular stomatitis in neonatal calves. *Arch Virol* 1982;71:251–258.
25. Roess AA, McCollum AM, Gruszynski K, et al. Surveillance of parapoxvirus among ruminants in Virginia and Connecticut. *Zoonoses Public Health* 2013;60:543–548.
26. Yaegashi G, Sasaki I, Chiba S, et al. Molecular analysis of parapoxvirus detected in eight calves in Japan. *J Vet Med Sci* 2013;75:1399–1403.
27. Inoshima Y, Murakami K, Yokoyama T, et al. Genetic heterogeneity among parapoxviruses isolated from sheep, cattle and Japanese serows (*Capricornis crispus*). *J Gen Virol* 2001;82:1215–1220.
28. Jeckel S, Bidewell C, Everest D, et al. Severe oesophagitis in an adult bull caused by bovine papular stomatitis virus. *Vet Rec* 2011;169:317.
29. Fraser CM, Savan M. Bovine papular stomatitis—a note on its diagnosis and experimental transmission in Ontario. *Can Vet J* 1962;3:107–111.
30. Kahrs RF. Papular stomatitis. In: Kahrs RF, eds. *Viral diseases of cattle*. 2nd ed. Ames, Iowa: Iowa State University Press, 2001;179–184.
31. Brown CC, Baker DC, Barker IK. Parapoxviral infections. In: Maxie MG, ed. *Pathology of domestic animals*. Vol 2. 5th ed. Philadelphia: Elsevier, 2007;162–163.
32. Sentsui H, Murakami K, Inoshima Y, et al. Isolation of parapoxvirus from a cow treated with interferon-γ. *Vet Microbiol* 1999;70:143–152.
33. Sentsui H, Inoshima Y, Minami A, et al. Survey on antibody against parapoxvirus among cattle in Japan. *Microbiol Immunol* 2000;44:73–76.