Table 8. The Comparison of Hepatitis Virus PCR with Routine Method

Age	Sex	Type of hepatitis	Qualitative PCR	Quantative PCR	Commercially available PCR*
79	F	HBV	positive	1.88×10^{9}	7.94×10^{7}
69	F	HBV	positive	4.39×10^{7}	2.51×10^{7}
57	M	HBV	positive	1.49×10^{8}	1.25×10^{8}
80	M	HBV	positive	1.59×10^{9}	1.26×10^{9}
79	M	HBV	positive	1.60×10^{8}	7.94×10^{6}
63	F	HBV	positive	2.90×10^{3}	5.01×10^{3}
75	M	HBV	negative	1.00×10^{1}	$<3.91\times10^{3}$
63	M	HCV	N.E.	2.51×10^{6}	2.00×10^{6}
70	F	HCV	N.E.	7.94×10^{4}	1.00×10^{5}
46	M	HCV	N.E.	3.98×10^{5}	2.51×10^{6}
80	M	HCV	N.E.	2.51×10^{6}	1.00×10^{7}
66	M	HCV	N.E.	5.01×10^{3}	1.00×10^{4}
71	F	HCV	N.E.	6.31×10^{5}	1.26×10^{6}
85	F	HCV	N.E.	2.51×10^{6}	6.31×10^{5}

HBV: hepatitis B, HCV: hepatitis C, N.E.: Not examined
*: performed by Special Research Laboratory, Hachioji, Japan

sidual giant proerythroblasts. A diagnosis of PRCA due to ParvoB19 was made, and the assay for ParvoB19 showed 6.9×10^{7} copies/mL in accordance with a positive qualitative PCR result and a positive serological IgM test for ParvoB19 (performed by SRL).

Discussion

The present study found a high incidence of viremia in patients with unexplained liver dysfunction and undetermined inflammation. The high proportion of hematologic malignancies including allogeneic bone marrow transplantation, that were underlying diseases in the patients included in this study may explain the high incidence of viremia. The multiplex PCR procedure appeared to be very useful in the clarification of uncertain liver dysfunction and inflammation. The patients in this cohort turned out to be highly immunodeficient and susceptible to viral infection. The identification of the high incidence of viremia may lead to better management of these patients.

TTV (22) was the most frequently detected virus in the present study; however, the relationship between TTV-positivity and the history of transfusion was unclear as shown in Table 4, thus suggesting a previous TTV infection in these TTV-positive patients. All patients in whom TTV was detected exhibited mild to moderate hepatitis, as observed in previous reports (22), except for patients 11 and 15. The lever dysfunction improved after a short time in these 2 patients, suggesting a transient exacerbation of TTV-related liver dysfunction by immunosuppressive treatment. This possibility, however, should be elucidated in the future in a larger cohort of immune-deficient patients because the relationship between the changes of liver dysfunction and TTV load was unclear in the follow-up examination. Similar incidences of HHV-6 viremia and TTV were observed in the present study. Apart from TTV that is widely distributed in the healthy population (22, 23), HHV-6 viremia is considered to be re-activation of this virus (24); therefore, this viremia indicates a severe immune-compromised condition. The high incidence of HHV-6 viremia in the present study could be explained by the high proportion of patients that underwent allogeneic or autologous hematopoietic stem cell transplantation or patients with hematologic malignancies, or AIDS. HHV-6 viremia occasionally advances to encephalitis or pneumonitis if its load is high (25). Therefore, identification of HHV-6 viremia is very important, and antiviral treatment is required. The present study, found that liver dysfunction was improved as the load of HHV-6 decreased in all 6 patients that were re-assayed for HHV-6 (Tables 4, 5). This suggests that liver dysfunction may be closely related to HHV-6 infection. While 2 of 17 patients in whom HHV-6 was detected showed normal liver function (Table 6), which is consistant with previous reports describing that hepatitis is not a major clinical manifestation of HHV-6 infection (26). In contrast, HHV-6-related hepatitis is reported in patients that underwent heart transplantation (27); therefore, the exact relationship between HHV-6 infection and hepatitis in immune-compromised patients should be clarified in the future. It was also important that the liver dysfunction acted as a proband that led to the discovery of serious viremia in the present study.

EBV and CMV infections can be categorized into 2 groups; primary infections that cause infectious mononucleosis (IM), and re-activation of both viruses. The present study, identified 2 patients with IM due to CMV (28) and 1 with EBV-IM (29). IM can be easily diagnosed because of its characteristic clinical picture. However, the multiplex PCR assay had definite advantages because it provided rapid results. The clinical significance of the re-activation of these 2 viruses is similar to that of HHV-6, and again in this situation, the assay system appeared to be highly useful.

There were some discrepancies regarding EBV detection between the results obtained by multiplex PCR and those obtained by routine serological tests as previously described. Patients 18 and 21, showed re-activation or primary infection patterns for both CMV and EBV when examined by routine serological methods, while EBV was not detected by the assay system. EBV-IM appeared to be atypical in these 2 patients because of the normal white cell count. Furthermore, cross-reactivity of EBV-specific IgM antibodies with CMV-antigens (30) or false positive EBV IgM serological tests (31) occasionally observed. Therefore, their IM may have been CMV-induced. EBV was detected in Patient 40, regardless of the previous infection pattern determined by the serological method. This patient had ulcerative colitis and was in an immune-deficient state. Persistent EBV viremia is likely in such conditions, and normal immune responses that produce the IgM antibody may be suppressed. Although the assay system appears to be reliable, further improvement of our system is necessary.

The authors state that they have no Conflict of Interest (COI).

References

1. Sugita S, Shimizu N, Watanabe K, et al. Use of multiplex PCR and real-time PCR to detect human herpes virus genome in ocular fluids of patients with uveitis. Br J Ophthalmol **92**: 928-932, 2008.
2. Gatto F, Cassina G, Broccolo F, et al. A multiplex calibrated real-time PCR assay for quantitation of DNA of EBV-1 and 2. J Virol Methods **78**: 98-105, 2011.
3. Burrows J, Nitsche A, Bayly B, Walker E, Higgins G, Kok T. Detection and subtyping of herpes simplex virus in clinical samples by LightCycler PCR, enzyme immunoassay and cell culture. BMC Microbiol **2**: 12, 2002, PMCID:PMC116582.
4. Espy MJ, Teo R, Ross TK, et al. Diagnosis of Varicella-Zoster virus infections in the Clinical laboratory by LightCycler PCR. J Clin Microbiol **38**: 3187-3189, 2000.
5. Schaade L, Kockelkorn P, Ritter K, Kleines M. Detection of cytomegalovirus DNA in human specimens by LightCycler PCR. J Clin Microbiol **38**: 4006-4009, 2000.
6. Ohyashiki JH, Suzuki A, Aritaki K, et al. Use of real-time PCR to monitor human herpesvirus 6 reactivation after allogeneic bone marrow transplantation. Int J Mol Med **6**: 427-432, 2000.
7. Boivin G, Côté S, Cloutier N, Abed Y, Maguigad M, Routy JP. Quantification of human herpesvirus 8 by real-time PCR in blood fractions of AIDS patients with Kaposi's sarcoma and multicentric Castleman's disease. J Med Virol **68**: 399-403, 2002.
8. Harder TC, Hufnagel M, Zahn K, et al. New LightCycler PCR for rapid and sensitive quantification of Parvovirus B19 DNA guides therapeutic decision-making in relapsing infections. J Clin Microbiol **39**: 4413-4419, 2001.
9. Whiley DM, Mackay IM, Sloots TP. Detection and differentiation of human polyomaviruses JC and BK by LigtCycler PCR. J Clin Microbiol **39**: 4357-4361, 2001.
10. Nishizawa T, Okamoto H, Konishi K, Yoshizawa H, Miyakawa Y, Mayumi M. A novel DNA virus (TTV) associated with elevated transaminase levels in posttransfusion hepatitis of unknown etiology. Biochem Biopsy Res Commun **241**: 92-97, 1997.
11. Corey L, Huang ML, Selke S, Wald A. Differentiation of herpes simplex virus types 1 and 2 in clinical samples by a real-time taqman PCR assay. J Med Viol **76**: 350-355, 2005.
12. Kimura H, Morita M, Yabuta Y, et al. Quantitative analysis of Epstein-Barr virus load by using a real-time PCR assay. J Clin Microbiol **37**: 132-136, 1999.
13. Piiparinen H, Helanterä I, Lappalainen M, et al. Quantitative PCR in the diagnosis of CMV infection and in the monitoring of viral load during the antiviral treatment in renal transplant patients. J Med Virol **76**: 367-372, 2005, PMID: 15902704.
14. Watzinger F, Suda M, Preuner S, et al. Real-time quantitative PCR assays for detection and monitoring of pathogenic human viruses in immunosuppressed pediatric patients. J Clin Microbiol **42**: 5189-5198, 2004.
15. Hara S, Kimura H, Hosino Y, et al. Detection of herpesvirus DNA in the serum of immunocompetent children. Microbiol Immunol **46**: 177-180, 2002, PMID:12008926.
16. Polstra AM, Van Den Burg R, Goudsmit J, Cornelissen M. Human herpesvirus 8 load in matched serum and plasma samples of patients with AIDS-associated Kaposi's sarcoma. J Clin Microbiol **41**: 5488-5491, 2003.
17. Herman J, Van Ranst M, Snoeck K, Beusekinck K, Lerut E, Van Damme-Lombaerts R. Polyomavirus infection in pediatric renal transplant recipients: evaluation using a quantitative real-time PCR technique. Pediatr Transplant **8**: 485-492, 2004.
18. Le Gal F, Gordien E, Affolabi D, et al. Quantification of hepatitis delta virus RNA in serum by consensus real-time PCR indicates different patterns of virological response to interferon therapy in chronically infected patients. J Clin Microbiol **43**: 2363-2369, 2005.
19. Jothikumar N, Cromeans TL, Robertson BH, Meng XJ, Hill VR. A broadly reactive one-step real-time RT-PCR assay for rapid and sensitive detection of hepatitis E virus. J Virol Methods **131**: 65-71, 2006, PMID: 16125257.
20. Künkel U, Höhne M, Berg T, et al. Quality control study on the performance of GB virus C/hepatitis G virus PCR. J Hepatol **28**: 978-984, 1998.
21. Moen EM, Sleboda J, Grinde B. Real-time PCR methods for independent quantitation of TTV and TLMV. J Viol Method **104**: 59-67, 2002.
22. Biagini P. Human circoviruses. Vet Microbiol **98**: 95-101, 2004.
23. Gallian P, Biagini P, Zhong S, et al. TT virus: a study of molecular epidemiology and transmission of genotypes 1, 2 and 3. J Clin Virol **17**: 43-49, 2000.
24. Zerr DN, Corey L, Kim HW, Huang ML, Nguy L, Borckh M. Clinical outcomes of human herpesvirus 6 reactivation after hematopoietic stem cell transplantation. Clin Infect Dis **40**: 932-940, 2005.
25. Ogata M, Satou T, Kawano R, et al. Correlations of HHV-6 viral load and plasma IL-6 concentration with HHV-6 encephalitis in allogeneic stem cell transplant recipients. Bone Marrow Transplant **45**: 129-136, 2010.
26. Zerr DM. Human herpesvirus 6: a clinical update. Herpes **13**: 20-24, 2006.
27. Robert C, Agut H, Lunel-Fabian F, Leger P. Human herpesvirus 6 infection and hepatitis following heart transplantation. Presse Med **23**: 1209-1210, 1994.
28. Han XY, Hellerstedt BA, Koller CA. Postsplenectomy cytomegalovirus mononucleosis is a distinct clinicopathologic syndrome. Am J Med Sci **339**: 395-399, 2010.
29. Vouloumanou EK, Rafailidis PI, Falagas ME. Current diagnosis and management of infectious mononucleosis. Curr Opin Hematol **19**: 14-20, 2012.
30. Lang D, Vornhagen R, Rothe M, Hinderer W, Sonneborn H-H, Plachter B. Cross-reactivity of Epstein-Barr virus-specific immunoglobulin M antibodies with cytomegalovirus antigens containing glycine homopolymers. Clin Diagn Lab Immunol **8**: 747-756, 2001.
31. Just-Nübling G, Korn S, Ludwig B, Stephan C, Doerr HW, Preiser W. Primary cytomegalovirus infection in an outpatient setting: laboratory markers and clinical aspects. Infection **31**: 318-323, 2003.

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Short Communication

Hemotropic mycoplasma infection in wild black bears (*Ursus thibetanus japonicus*)

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ABSTRACT

This is the first report on *Mycoplasma* infection in wild bears. We report a novel hemotropic *Mycoplasma* (also called hemoplasma) detected in a free-ranging black bear (*Ursus thibetanus japonicus*) in Japan. We then used real-time PCR to look for hemoplasma DNA in blood samples collected from 15 bears and found that eight (53%) were positive. Among these eight PCR samples, seven showed a melting temperature of around 85.5 °C, while the remaining one showed a single peak at 82.26 °C. Almost the entire region of the 16S rRNA gene as well as the 16S–23S rRNA intergenic transcribed spacer (ITS) region from the sample that showed a melting temperature of 82.26 °C was successfully amplified by means of end-point PCR. The nucleotide sequences of the 16S rRNA gene and the ITS region were then determined and compared with those of authentic *Mycoplasma* species. Our examinations revealed the presence of a novel hemoplasma in Japanese black bears.

1. Introduction

Hemoplasma is an informal name for a group of hemotropic prokaryotes that lack a cell wall and have never been cultured *in vitro* (Messick, 2004). This group is composed of species formerly classified as *Eperythrozoon* and *Hemobartonella* as well as newly identified hemotropic mycoplasmas. Hemoplasmas have been demonstrated in various mammalian species, but never previously in bears. Therefore we examined hemoplasma infection in Japanese black bears (*Ursus thibetanus japonicus*), a species in the family *Ursidae* of the order *Carnivora*. Japanese black bears live in the forests of Honshu and Shikoku islands and are legally hunted, despite the absence of any definitive population estimate. Here we report on a novel hemoplasma in a wild bear that we demonstrated by analyzing the 16S rRNA gene and the 16S–23S rRNA intergenic transcribed spacer (ITS) region.

2. Materials and methods

2.1. Blood collection

Anti-coagulated blood samples were collected from slash wounds in carcasses of 15 wild Japanese black bears killed by licensed hunters in Iwate prefecture in 2011, and stored at −20 °C until examination. Gender and age of bears were not recorded in this study.

2.2. DNA extraction

Total DNA was extracted from 200 μl blood samples collected from bear carcasses using the QIAamp DNA Blood

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Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Negative controls consisting of 200 μl phosphate-buffered saline solution were prepared with each batch. Extracted DNA specimens were subjected to concentration measurements in a GeneQuant II spectrophotometer (GE Healthcare, Tokyo, Japan) and stored at −80 °C prior to examination.

2.3. Real-time PCR

For preliminary screening of hemoplasma infection, specific PCR primers (forward primer: 5′-ATATTCCTACGGGAAGCAGC-3′ equivalent to nucleotide numbers 328–347 of *Mycoplasma wenyonii* [AY946266] and reverse primer: 5′-ACCGCAGCTGCTGGCACATA-3′ equivalent to nucleotide numbers 503–522 of *M. wenyonii*) for the 16S rRNA gene of hemoplasmas were used in real-time PCR assay as described previously (Nishizawa et al., 2010). Real-time PCR was performed in a SmartCycler instrument (Cepheid, Sunnyvale, CA, USA) with SYBR Premix Ex Taq (Code #RR041A, TaKaRa Bio., Shiga, Japan). The reaction mixture contained 1 μl of each primer (10 pmol/μl), 12.5 μl of 2× premix reaction buffer and water to a volume of 23 μl. Finally, 2 μl (200 pg) of a DNA sample as a template was added to this mixture. Amplification was achieved with 40 cycles of denaturation at 95 °C for 5 s, renaturation at 57 °C for 20 s, and elongation at 72 °C for 15 s, after the initial denaturation at 94 °C for 30 s. Fluorescence readings in a channel for SYBR Green I were taken throughout the experiments. Sensitivity and specificity of this real-time PCR have been confirmed previously (Harasawa et al., 2005). After real-time PCR, the melting experiment was performed from 60 to 95 °C at 0.2 °C/s with a smooth curve setting averaging one point. Melting peaks were visualized by plotting the first derivative against the melting temperature (T_m) as described previously (Harasawa et al., 2005). The T_m was defined as a peak of the curve; if the highest point was a plateau, then the mid-point was identified as the T_m.

2.4. End-point PCR for the 16S rRNA gene

The positive samples from the real-time PCR experiment were further subjected to end-point PCR to amplify the entire region of the 16S rRNA gene. End-point PCR was carried out with 50-μl reaction mixtures each containing 1 μl of DNA solution, 0.5 μl of TaKaRa LA Taq™ (5 units/μl), 5 μl of 10× LA PCR™ Buffer II, 8 μl of 25 mM $MgCl_2$ (final 4.0 mM), 8 μl of dNTP mixture (2.5 mM each), 0.2 μl each of forward primer (5′-AGAGTTTGATCCTGGCTCAG-3′, equivalent to nucleotide numbers 11–30 of *M. wenyonii* [AY946266], or 5′-ATATTCCTACGGGAAGCAGC-3′, equivalent to nucleotide numbers 328–347 of *M. wenyonii*) and reverse primer (5′-ACCGCAGCTGCTGGCACATA-3′, equivalent to nucleotide numbers 503–522 of *M. wenyonii*, or 5′-TACCTTGTTACGACTTAACT-3′, equivalent to nucleotide numbers 1446–1465 of *M. wenyonii*) (50 pmol/μl each), and water to a final volume of 50 μl. After the mixture was overlaid with 20 μl of mineral oil, the reaction cycle was carried out 35 times with denaturation at 94 °C for 30 s, annealing at 58 °C for 120 s and extension at 72 °C for 60 s in a thermal cycler.

2.5. End-point PCR for ITS region

Next, we amplified the ITS region of this specimen by end-point PCR using forward primer Hemo16-23S-F (5′-GTTCCCAGGTCTTGTACACA-3′) and reverse primer Hemo16-23S-R1 (5′-CAGTACTTGTTCACTGGTA-3′) as described previously (Harasawa et al., 1996).

2.6. Sequencing of PCR products

Both the end-point PCR products from the 16S rRNA gene and the ITS region were fractionated on horizontal, submerged 1.0% SeaKem ME agarose gels (FMC Bioproducts, Rockland, ME, USA) in TAE buffer (40 mM Tris, pH 8.0, 5 mM sodium acetate, 1 mM disodium ethylenediaminetetracetate) at 50 V for 60 min. After electrophoresis, the gels were stained in ethidium bromide solution (0.4 μg/ml) for 15 min and visualized under a UV transilluminator. DNA was extracted using a NucleoSpin Extract II kit (Macherey-Nagel, Düren, Germany) and was subjected to direct sequencing in a 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

2.7. Nucleotide sequence accession number

The nucleotide sequence consisting of the 16S rRNA gene and the ITS region combined has been deposited in the DDBJ, EMBL, GSDB and NCBI nucleotide sequence databases under the accession number AB725596.

2.8. Phylogenetic analysis

The nucleotide sequence of the 16S rRNA gene from the bear strain along with the 21 established mycoplasma species was aligned using CLUSTAL W (Thompson et al., 1994) with further adjustment made manually as necessary. A phylogenetic tree was generated using the neighbor-joining method (Saitou and Nei, 1987) from a distance matrix corrected for nucleotide substitutions by the Kimura two-parameter model (Kimura, 1980).

2.9. Secondary structure of ITS region

The secondary structures of the ITS region were predicted according to the algorithm of Zuker and Stiegler (1981), since secondary structures of this region have sometimes provided key information used to distinguish closely related mycoplasma species (Harasawa et al., 1996, 2000; Harasawa and Kanamoto, 1999).

3. Results

3.1. Detection of hemoplasmas

Out of the 15 blood samples tested with real-time PCR, eight (53%) were found provisionally positive for hemoplasmas. Among these eight samples, seven showed a T_m around 85.5 °C, while the remaining one showed a single peak at 82.26 °C (Fig. 1).

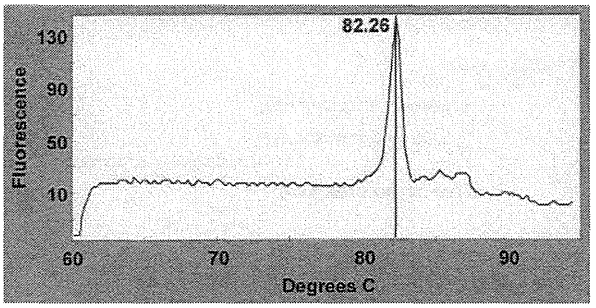

Fig. 1. Thermal melting curve of the PCR products visualized with depicted SYBR Green I. A characteristic melting temperature at 82.26 °C was evident in one bear hemoplasma strain.

3.2. End-point PCR of 16S rRNA gene and ITS

Of the eight positive samples, only the one that showed a T_m at 82.26 °C produced a clearly visible band in both end-point PCR procedures and was subjected to direct sequencing. Attempts to amplify larger fragments of the 16S rRNA gene and the ITS region were unsuccessful from the remaining seven samples.

3.3. Nucleotide sequences of 16S rRNA gene and ITS

We determined the nucleotide sequences of almost the entire region of the 16S rRNA gene and the ITS region for the hemoplasma strain detected in bears and compared them with those of other mycoplasmas by constructing a phylogenetic tree.

3.4. Phylogenetic analysis

As shown in Fig. 2, a phylogenetic tree for the 16S rRNA gene constructed by the algorithms in the PHYLIP program (DDBJ, Shizuoka, Japan) using the neighbor-joining method indicated that the hemoplasma strain detected in bears was closely related to *M. haemofelis* and *M. haemocanis*, with homology levels higher than 98%. The dataset was resampled 1000 times to generate bootstrap values.

In a phylogenetic tree based on the ITS region, on the other hand, the bear strain was distinct from previously

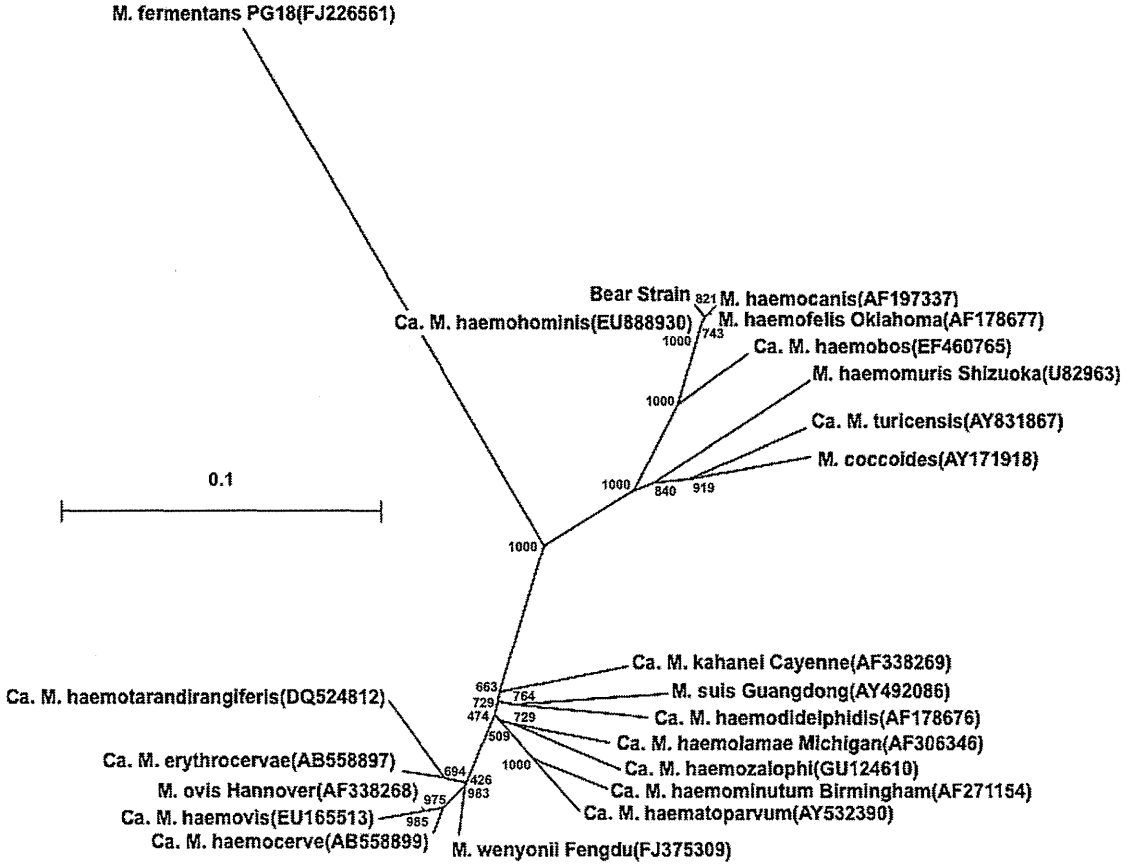

Fig. 2. A neighbor-joining phylogenetic tree based on a 16S rRNA gene comparison among mycoplasmas including 21 hemoplasma species (accession numbers are given in parentheses) inclusive of a putative taxon created by a bear strain. *M. fermentans* was included as an out-group. Genetic distances were compared with Clustal W (Thompson et al., 1994). Numbers at the branch points refer to the values of bootstrap probability of 1000 replications. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances that were used to infer the phylogenetic tree (scale bar, 0.1 nucleotide substitutions per site).

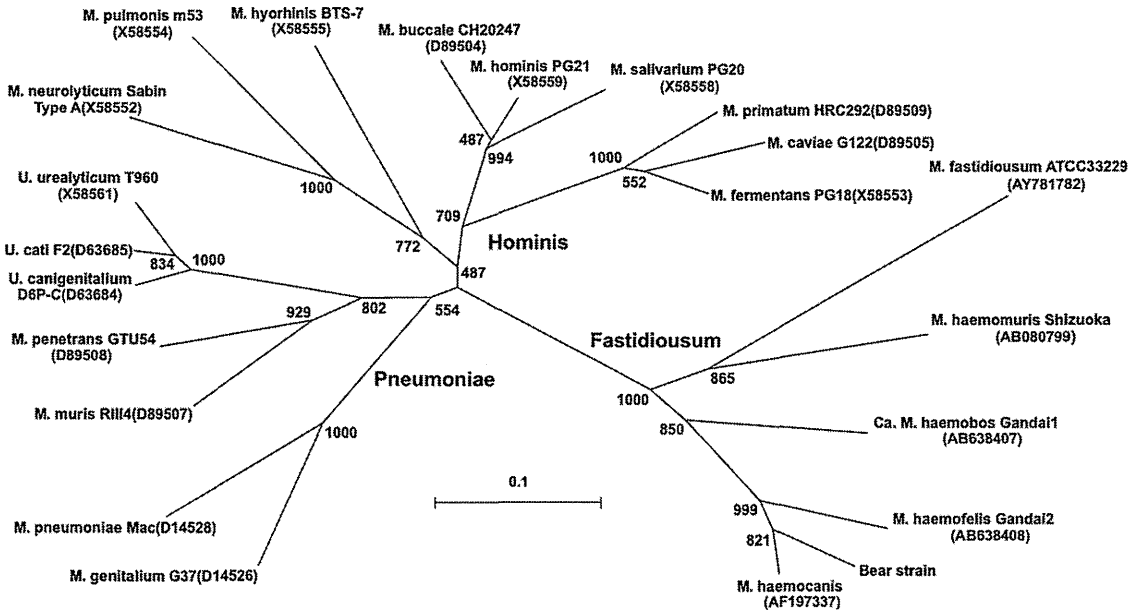

Fig. 3. Unrooted phylogenetic tree based on ITS comparison, suggesting a monophyletic relationship among hemoplasmas and *M. fastidiosum*. The nucleotide sequences were obtained from the DNA databases and accession numbers are given in parenthesis.

described authentic *Mycoplasma* species including *M. haemofelis* and *M. haemocanis* (Fig. 3).

3.5. Secondary structure of ITS

Five stem-loop domains were predicted in the ITS region of the bear hemoplasma strain (Fig. 4). The boxA was assigned on the stem portion of domain IV.

4. Discussion

The nucleotide sequence of the 16S rRNA gene has been widely used in microbiology for identifying uncultivable microorganisms as new species. 16S rRNA gene sequences have also been the basis for reclassification of hemotropic *Mycoplasma* species (Neimark et al., 2001, 2002). Hemoplasmas are divided into two phylogenetic clusters, Wenyonii (or Heamominutum) and Haemofelis (Peters et al., 2008; Watanabe et al., 2010). In the present study, phylogenetic analysis of the 16S rRNA gene indicated that the bear strain belonged in the Haemofelis cluster. The bear strain showed 98% homology with *M. haemofelis*, a level higher than the cut-off for species differentiation (Drancourt and Raoult, 2005). This may be due to a close genealogical relationship among the host animals in the order *Carnivora* for these hemoplasma species.

Because 16S rRNA sequences may not be sufficient to determined a species' taxonomic status, we also examined the ITS region of the bear strain (Fox et al., 1992). *M. haemocanis* and *M. haemofelis* are recognized as distinct species due to a difference in their *rnpB* genes (Birkenheuer et al., 2002), though they have 99.2% homology between their 16S rRNA gene sequences. The ITS region of the genus *Mycoplasma* is well conserved across species and is used as a complementary genetic marker for identification and classification of mycoplasmas as well as other bacterial species (Graham et al., 1997; Harasawa et al., 1996; Jagoueix et al., 1997; Thain et al., 1977; Volokhov et al., 2012). The nucleotide sequence of the ITS region of the bear hemoplasma strain was compared with those of 20 other *Mycoplasma* species in a phylogenetic analysis. The resulting comprehensive phylogenetic tree suggested that these mycoplasma sequences were divided into three clusters, Hominis, Pneumoniae and Fastidiosum, as described previously (Sasaoka et al., 2012). Hemoplasmas are included in the Fastidiosum clade, and thus are given a more precise affiliation than that permitted by phylogenetic analysis based on 16S rRNA sequences (Neimark et al., 2001, 2002). Although the Hominis and Pneumoniae groups of mycoplasmas have already been established through phylogenetic analysis of the 16S rRNA gene (Peters et al., 2008), we showed a monophyletic relationship among hemoplasmas and *M. fastidiosum* by phylogenetically analyzing the ITS regions.

The secondary structures of the ITS region were predicted according to the algorithm of Zuker and Stiegler (1981), since secondary structures of this region have sometimes provided information that is key to distinguishing closely related species among mycoplasmas (Harasawa et al., 1996). Five stem-loop domains were predicted in the ITS region of the bear hemoplasma strain (Fig. 3). The boxA was assigned on the stem portion of domain IV, as is common to other hemoplasmas (Sasaoka et al., 2012). The boxA, originally found at upstream regions of the *nut* site of the lambda phage genome, is considered to be a *cis*-acting element for the *Escherichia coli* NusA protein, a transcription control factor (Olson et al., 1987). The boxB sequence previously reported in mycoplasmas (Harasawa et al., 1992) was also assigned to a location about ten nucleotides upstream of the boxA

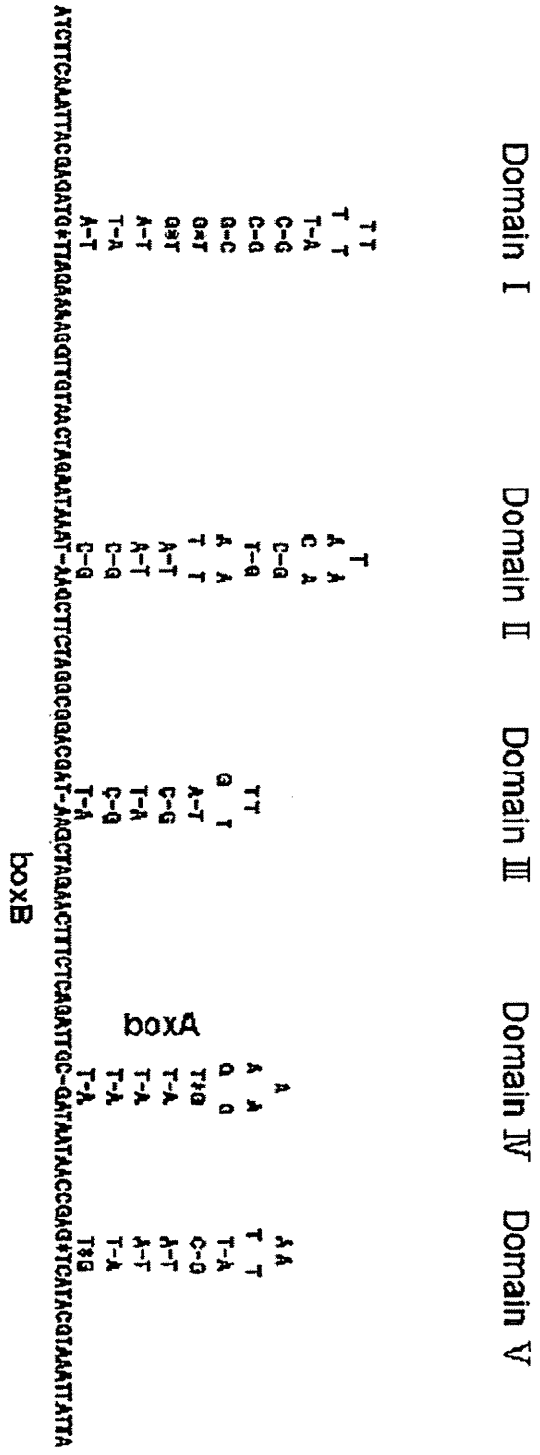

Fig. 4. Hypothetical secondary structures for ITS of the bear strain. Canonical Franklin–Watson–Crick base-pairing is indicated by hyphens, and a wobble base-pairing tolerated in the secondary structure is shown by an asterisk. The boxA was a part of the stem region of domain IV, and the boxB was located between domains III and IV.

sequence of the bear hemoplasma strain. The presence of these particular motifs suggests that ITS might act as a controlling element for transcription of the rRNA operon in hemoplasmas. The spacer tRNA gene reported in the genus *Acholeplasma* (Nakagawa et al., 1992) was not identified in the ITS of the bear hemoplasma; its absence is a common feature among the other species of the genus *Mycoplasma* (Uemori et al., 1992). Although transposase gene has been demonstrated in the ITS of *M. imitans* (Harasawa et al., 2004), it is not found in the bear hemoplasma species.

Here we have demonstrated a previously unreported hemoplasma species in wild Japanese black bears. This species was closely related to *M. haemofelis* and *M. haemocanis* in a phylogenetic tree based on nucleotide sequences of the 16S rRNA gene, but was distinct from other established mycoplasma species in terms of the primary and secondary structures of the ITS region. The pathogenic traits of this hemoplasma species in bears remain unexplored.

Conflict of interest

The authors of this paper have no conflict of interest that could inappropriately influence or bias the paper.

References

Birkenheuer, A.J., Breitschwerdt, E.B., Alleman, A.R., Pitulle, C., 2002. Differentiation of *Haemobartonella canis* and *Mycoplasma haemofelis* on the basis of comparative analysis of gene sequence. Am. J. Vet. Res. 63, 1385–1388.

Drancourt, M., Raoult, D., 2005. Sequence-based identification of new bacteria: a proposition for creation of an orphan bacterium repository. J. Clin. Microbiol. 43, 4311–4315.

Fox, G.E., Wisotzkey, J.D., Jurtshuk Jr., P., 1992. How close is close: 16S rRNA sequence identity may not be sufficient to guarantee species identity. Int. J. Syst. Bacteriol. 42, 166–170.

Graham, T.A., Golsteyn-Thomas, E.J., Thomas, J.E., Gannon, V.P.J., 1997. Inter- and intraspecies comparison of the 16S–23S rRNA operon intergenic spacer regions of six *Listeria* spp. Int. J. Syst. Bacteriol. 47, 863–869.

Harasawa, R., Hotzel, H., Sachse, K., 2000. Comparison of the 16S-23S rRNA intergenic spacer regions among strains of the Mycoplasma mycoides cluster, and reassessment of the taxonomic position of Mycoplasma sp. bovine group 7. Int. J. Syst. Evol. Microbiol. 50, 1325–1329.

Harasawa, R., Kanamoto, Y., 1999. Differentiation of two biovars of Ureaplasma urealyticum based on the 16S-23S rRNA intergenic spacer region. J. Clin. Microbiol. 37, 4135–4138.

Harasawa, R., Lefkowitz, E.J., Glass, J.I., Cassell, G.H., 1996. Phylogenetic analysis of the 16S–23S rRNA intergenic spacer region of the genus *Ureaplasma*. J. Vet. Med. Sci. 58, 191–195.

Harasawa, R., Mizusawa, H., Fujii, M., Yamamoto, J., Mukai, H., Uemori, T., Asada, K., Kato, I., 2005. Rapid detection and differentiation of the major Mycoplasma contaminants in cell cultures using real-time PCR with SYBR Green I and melting curve analysis. Microbiol. Immunol. 49, 859–863.

Harasawa, R., Pitcher, D.G., Ramirez, A.S., Bradbury, J.M., 2004. A putative transposase gene in the 16S–23S rRNA intergenic spacer region of *Mycoplasma imitans*. Microbiology 150, 1023–1029.

Harasawa, R., Uemori, T., Asada, K., Kato, I., Shiragami, N., 1992. 'boxA'-like sequence between the 16S/23S spacer in the rRNA operon. FEBS Lett. 297, 209–211.

Jagoueix, S., Bove, J.M., Garnier, M., 1997. Comparison of the 16S/23S ribosomal intergenic regions of '*Candidatus liberobacter asiaticum*' and '*Candidatus liberobacter africanum*' the two species associated with citrus huang-longbing (greening) disease. Int. J. Syst. Bacteriol. 47, 224–227.

Kimura, M., 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. 16, 111–120.

Messick, J.B., 2004. Hemotrophic mycoplasmas (hemoplasmas): a review and new insights into pathogenic potential. Vet. Clin. Pathol. 33, 2–13.

Nakagawa, T., Uemori, T., Asada, K., Kato, I., Harasawa, R., 1992. *Acholeplasma laidlawii* has tRNA genes in the 16S–23S spacer of the rRNA operon. J. Bacteriol. 174, 8163–8165.

Neimark, H., Johansson, K.-E., Rikihisa, Y., Tully, J.G., 2001. Proposal to transfer some members of the genera Haemobartonella and Eperythrozoon to the genus *Mycoplasma* with descriptions of '*Candidatus mycoplasma haemofelis*' '*Candidatus mycoplasma haemomuris*', '*Candidatus mycoplasma haemosuis*' and '*Candidatus mycoplasma wenyonii*'. Int. J. Syst. Evol. Microbiol. 51, 891–899.

Neimark, H., Johansson, K.-E., Rikihisa, Y., Tully, J.G., 2002. Revision of haemotrophic Mycoplasma species names. Int. J. Syst. Evol. Microbiol. 52, 683.

Nishizawa, I., Sato, M., Fujihara, M., Sato, S., Harasawa, R., 2010. Differential detection of hemotropic Mycoplasma species in cattle by melting curve analysis of PCR products. J. Vet. Med. Sci. 72, 77–79 Erratum J. Vet. Med. Sci. 72, 1704.

Olson, E.R., Tomich, C.-S., Friedman, D.I., 1987. The nusA recognition site. J. Mol. Biol. 180, 1053–1063.

Peters, I.R., Helps, C.R., McAuliffe, L., Neimark, H., Lappin, M.R., Gruffydd-Jones, T.J., Day, M.J., Hoelzle, L.E., Willi, B., Meli, M., Hofmann-Lehmann, R., Tasker, S., 2008. RNase P RNA gene (*rnpB*) phylogeny of hemoplasmas and other Mycoplasma species. J. Clin. Microbiol. 46, 1873–1877.

Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425.

Sasaoka, F., Suzuki, J., Fujihara, M., Watanabe, Y., Nagai, K., Harasawa, R., 2012. Examination of the 16S–23S rRNA intergenic spacer sequences of '*Candidatus mycoplasma haemobos*' and *Mycoplasma haemofelis*. J. Vet. Med. Sci. 74, 83–87.

Thain, T., Ward-Rainey, N., Kroppenstedt, R.M., Stackebrandt, E., Rainey, F.A., 1977. Discrimination of *Streptomyces albidoflavus* strains based on the size and number of 16S–23S ribosomal DNA intergenic spacers. Int. J. Syst. Bacteriol. 47, 202–206.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.

Uemori, T., Asada, K., Kato, I., Harasawa, R., 1992. Amplification of the 16S–23S spacer region in rRNA operons of mycoplasmas by the polymerase chain reaction. Syst. Appl. Microbiol. 15, 181–186.

Volokhov, D.V., Simonyan, V., Davidson, M.K., Chizhikov, V.E., 2012. RNA polymerase beta subunit (*rpoB*) gene and the 16S–23S rRNA intergenic transcribed spacer region as complementary molecular markers in addition to the 16S rRNA gene for phylogenetic analysis and identification of the species of the family Mycoplasmataceae. Mol. Phylogenet. Evol. 62, 515–528.

Watanabe, Y., Fujihara, M., Obara, H., Matsubara, K., Yamauchi, K., Harasawa, R., 2010. Novel hemoplasma species detected in free-ranging sika deer (*Cervus nippon*). J. Vet. Med. Sci. 72, 1527–1530.

Zuker, M., Stiegler, P., 1981. Optimal computer folding of large RNA sequences using thermodynamics and auxiliary. Nucleic Acids Res. 9, 133–148.

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Research Article Open Access

Serological Survey to Determine the Occurrence of Blue Tongue Virus, Bovine Leukemia Virus and Herpesvirus Infections in the Japanese Small Ruminant Population from Northern Districts

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Abstract

Ovine sera, collected from the northern prefectures of Hokkaido, Aomori and Iwate in Japan, were examined for the presence of antibodies against Blue tongue virus (BTV), bovine leukemia virus (BLV), bovine *Herpesvirus* type 1, agent of infectious bovine rhinotracheitis (IBR), ovine *Herpesvirus* type 2 (OvHV-2), agent of sheep-associated malignant catarrhal fever (SA-MCF), and bovine *Herpesvirus* type 4 (BoHV-4), using agar immune diffusion, serum neutralisation (SN) and enzyme linked immunosorbent assay (ELISA) tests. No animals were positive to IBR, BoHV-4 or BLV antigens. Antibodies against BTV have been detected in 3 samples (1.11%) in two flocks from Hokkaido. The seroprevalence of OvHV-2 was observed in twelve flocks from the 3 considered prefectures, in 56 sheep and two goats, with 37.66% of samples giving a positive reaction in the serum neutralization test. The infections did not appear to be related to the reduction in sheep productivity. Immune reaction reported in goats could refer to Caprine *Herpesvirus*-2 (CpHV-2). These results indicate that sheep are reservoirs for OvHV-2 in the field in Japan.

Keywords: Blue tongue ; Bovine *herpesvirus* type 4 ; Bovine leukemia virus; Infectious bovine rhinotracheitis; Japan ; Malignant catarrhal fever ; Small ruminants

Introduction

In Japan, bovine farming represents an important economical resource, with production excellences like black Japanese cattle breed, producing Kobe beef, while ovine farming is a relatively minor sector, and population is constituted by approximately 10,000 heads [1]. In some farms, cows are housed close to sheep pens or have access to common pastures. Such close contacts may represent a potential role in the diffusion of pathogenic agents to valuable cattle breeds.

Pathogens affecting cattle welfare and health have been accurately investigated in Japan. However, until now, only scarce information is available on epidemiology of virus pathogens in sheep. Among relevant pathogens, Blue tongue virus (BTV) was reported in cattle from Tochigi prefecture in 1994 [2] and in 2001 [1], and from Hiroshima and Fukushima prefectures in 2005 and 2006 [1]. Studies on bovine leukemia virus (BLV) demonstrated the occurrence of the disease in cattle [3]. Concerning *Herpesvirus* infections, studies undertaken in Japan on bovine *Herpesvirus* type 1 (BoHV-1), agent of infectious bovine rhinotracheitis (IBR), pathogen of worldwide importance affecting primarily cattle, showed the diffusion in cattle population [4,5]. Similarly, studies on ovine *Herpesvirus* type 2 (OvHV-2), known cause of the sheep-associated malignant catarrhal fever (SA-MCF) [6,7], and on bovine *Herpesvirus* type 4 (BoHV-4) demonstrated the occurrence of the diseases in cattle in the country [3,8].

Concerning small ruminants, sheep are naturally sensible to BTV and *Herpesvirus* infections (BoHV-1, OvHV-2 and BoHV-4) [9-11]. In particular, sheep are natural reservoir of OvHV-2. Generally, as other natural reservoir species for the viruses causing MCF, sheep does not exhibit any clinical signs of infection [10]. However, if infected with very high doses of virus, sheep can develop a mild form of MCF [12,13]. Domestic goats harbor their own closely-related strain of MCF virus. It has been termed caprine *Herpesvirus*-2 (CpHV-2) [14]. Although natural BLV infection occurs only in cattle, water buffaloes, and capybaras, sheep are highly susceptible to infection by inoculation of the virus, with a persistent antibody response, and develop tumours more often and at a younger age than cattle [15]. Therefore, risk of iatrogenic contamination has to be considered.

BTV was reported in sheep from Tochigi prefecture in 1994 (77 serologically positive animals) [2] and in 2001 (9 affected animals) [1]. Only one previous report showed prevalence of OvHV-2 in sheep in Japan [16], which described a seroprevalence of 64.3% in 238 sheep samples originated from 10 farms from Hokkaido. No further investigation has been reported. No previous epidemiological surveys on BLV, BoHV-1 or BoHV-4 have been undertaken in small ruminants in Japan. Furthermore, no clinical cases due to these infections have been reported among sheep flocks.

In order to explore the presence of BTV, BLV, and *Herpesvirus* (BoHV1, OvHV-2 and BoHV-4) infections in sheep, and to obtain a preliminary picture of their epidemiology among the Japanese sheep population, a serological survey was carried out between September 2007 and January 2008. These samples came from the northern prefectures in Japan, Hokkaido, Aomori and Iwate, as they have the most part of small ruminant population, approximately more than 50% in 2007 [1].

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Materials and Methods

The survey was performed on sheep raised both commercially and traditionally, from farms with a limited number of animals up to large flocks of 700 heads. The number of flocks was determined according to the animal population of each prefecture and is representative of the livestock production systems in the country. Ten flocks were sampled in Hokkaido prefecture, considering that about 37% of sheep breeding in Japan is concentrated in this region [1]. The ten flocks were arbitrarily chosen to include flocks from different regions of Hokkaido prefecture. The sampling was completed with four flocks from Tohoku area, two from Iwate prefecture and two from Aomori prefecture (Figure 1 and Table 1).

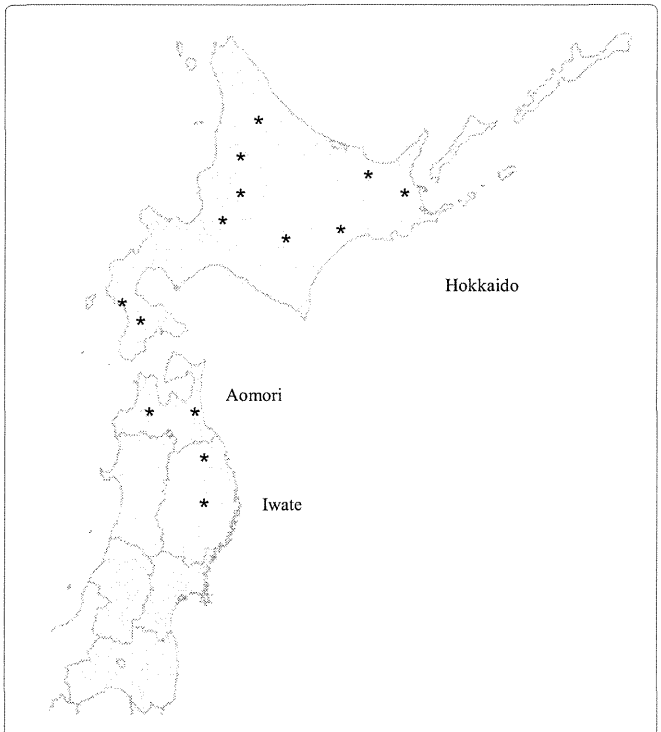

Figure 1: Northern Prefectures of Japan. Grey line: Prefecture boundaries; Light grey line: municipality boundaries; * places of sampling in Hokkaido, Aomori and Iwate Prefectures.

A maximum of 20 animals were sampled from each flock selected for sampling, according to the national standard of flock composition (number of rams, ewes and yearlings). In two of the 14 flocks, only 11 and 16 sheep, respectively, were available for sampling, and in one flock five goats were additionally sampled, and therefore, a total of 272 serum samples were collected. All age categories, from one year to 12 years of age, were sampled. Lambs were not sampled to avoid interpretation difficulties due to the potential presence of maternal antibodies. The majority of the sampled animals were Suffolk and Suffolk cross-breed. Other breeds were represented by Romanov, Cheviot, Corriedale, Friesland, Black Welsh Mountain and Poll Dorset sheep. Goats were cross-breed Shibayaghi x Tokara x Dane. All the sera were stored at −20°C prior to examination. The collected ovine sera were subsequently transported to laboratories in Italy for further analyses, under the authorization permitted by the Ministry of Health, Rome, Italy.

Serological testing for OvHV-2 and BoHV-4 was carried out using serum neutralisation (SN) test. In a 96-well plate, heat-inactivated serum samples were diluted from an initial dilution of 1:2 to 1:256, in double, and placed in contact with 100 $TCID_{50}$ of previously titrated *Alcelaphine herpesvirus 1* WC-11 strain or BoHV-4 Movar 33/63 strain. After incubation for 1 h at 37°C under 5% CO_2 to enable viral neutralisation, 5×10^5/ml of bovine turbinate cells (Madin-Darby bovine kidney for BoHV-4) suspended in minimum essential medium (MEM) (Eurobio, France). The medium contains penicillin 100 IU/ml, streptomycin 100 µg/ml, gentamicin 5 µg/ml, nystatin 50 IU/ml and 10% fetal calf serum (FCS) (Sigma, Germany), was added to each well. After 5 days, the cytopathic effect (CPE) in the wells was evaluated and the antibody titre was defined as the highest serum dilution able to inhibit at least 75% of the CPE. The positive and negative reference sera, cells and virus controls (Istituto Zooprofilattico Sperimentale, dell'Abruzzo e del Molise 'G. Caporale', IZSA&M, Teramo, Italy), were included in each plate.

Flock	Prefecture	Flock characteristics			Sampled animals		
		Number of animals	Flock composition	Breeds	Number	Gender	
						Rams	Ewes
1	Hokkaido	50	5 rams, 35 ewes, 10 yearlings	Cross breed, Suffolk, Cheviot, Romanov, Black, South down and Corriedale	20	0	20
2	Hokkaido	84	4 rams, 47 ewes, 33 yearlings	Suffolk, some South down and Black	20	0	20
3	Hokkaido	410	10 rams, 200 ewes, 200 yearlings	Suffolk	20	0	20
4	Hokkaido	178	4 rams, 80 ewes, 94 yearlings	Suffolk	20	3	17
5	Hokkaido	600	13 rams , 270 ewes, 30 yearlings, 287 lambs	Poll dorset, cross breeds Suffolk x Cheviot	20	1	19
6	Hokkaido	221	1 ram, 80 ewes, 40 yearlings, 100 lambs	Cross breeds South down x Poll dorset	20	0	20
7	Hokkaido	82	2 rams, 29 ewes, 14 yearlings, 37 lambs	Suffolk and Suffolk cross breed	20	0	20
8	Hokkaido	700	260 ewes, 440 yearlings	Cross breed, Romanov, Poll dorset, Suffolk	20	0	20
9	Hokkaido	100	2 rams, 40 ewes, 58 yearlings	Cross breed, Suffolk, Romanov, Friesland, Black welsh mountain	20	12	8
		100 goats		Cross breed Shibayaghi / Tokara / Dane	5		
10	Hokkaido	35	25 ewes, 10 lambs	Corriedale	11	8	3
11	Iwate	34	2 rams, 24 ewes, 8 yearlings	Cross breed, Suffolk, Romanov, Cheviot, Corriedale	20	1	19
12	Iwate	52	1 ram, 30 ewes, 21 yearlings	Suffolk	20	1	19
13	Aomori	34	1ram, 26 ewes, 7 yearlings	Suffolk	20	0	20
14	Aomori	22	22 ewes	Suffolk	16	0	16
Total					272	26	241

Table 1. Details of flocks sampled for serological testing of antibodies against pathogens in sheep from prefectures of northern Japan. Data show the number of samples.

Screening for anti - BLV antibodies was performed using an agar gel immunodiffusion (AGID) (a prescribed test for international trade) in accordance with the Manual of diagnostic tests and vaccines for terrestrial animals of the World Organisation for Animal Health (*Office International des Épizooties*: OIE) [15]. Antigen and control sera were obtained from the IZSA&M, Teramo, Italy.

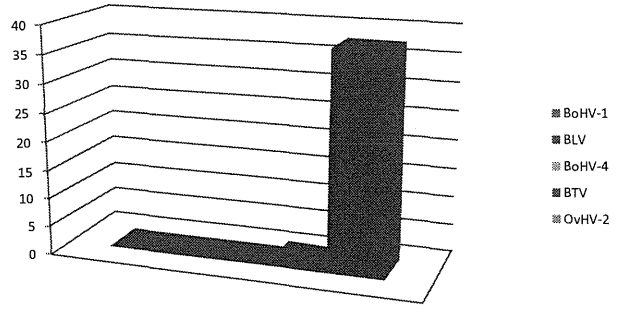

Figure 2: Summarized results of serological screening for BoHV-1 (IBR), BLV, BTV, BoHV-4 and OvHV-2 in sheep from Prefectures of Northern Japan. None of the tested animals resulted serologically positive for BLV, BoHV-1 and BoHV-4 antigens. Antibodies against BTV have been detected in 3 samples (1.11%), in two flocks from Hokkaido. A seroprevalence of 37.66% for anti-OvHV-2 immunoglobulins was detected from the three considered Prefectures.

	Positive	% Positive*	Negative	NE	Toxic	Total
IBR (ELISA)	0	0	272	-	-	272
BLV (AGID)	0	0	272	-	-	272
BTV (ELISA)	3	1.11	272	-	-	272
BoHV-4 (SN)	0	0	208	64	-	272
OvHV-2 (SN)	58	37.66	96	38	80	272

NE: not executed due to insufficient aliquots for testing

*: percentage computed excluding samples resulting toxic or not tested for insufficient serum quantity

Table 2. Results of serological screening for antibodies to IBR, BLV, BTV, BoHV-4 and OvHV-2 in sheep from Prefectures of Northern Japan.

Serological testing for antibodies against BoHV-1 glycoprotein B was performed by enzyme linked immunosorbent assay (ELISA), using a commercial kit (IDEXX IBR gB, IDEXX, USA), following the manufacturers' instructions.

Antibodies against BTV were detected using a competitive ELISA kit developed by the National Reference Centre for Exotic Diseases (IZSA&M, Teramo, Italy) [17].

Farmers were interviewed regarding flock management, productivity and losses, referring also to previous years. Concerning flock production, the annual lambing rate was calculated as number of lambs born per ewes exposed to the ram and it was based on a lambing season occurring from February to April, with an exception being made for one farm where the reproductive cycle was related to 3 breeding seasons. In order to evaluate a possible relationship between the prevalence of infection and production parameters such as annual lambing rate, annual lamb mortality rate and annual adult mortality rate, the animals were compared for their screened pathogens infection rate proportions by computing Pearson's correlation coefficients statistics. Differences were considered to be significant at $P<0.05$.

Results

Results of serological screening for antibodies to BTV, BLV, BoHV-1 (IBR), OvHV-2 and BoHV-4 in sheep from prefectures of northern Japan are summarized in Figure 2 and Table 2. All the 272 sera were submitted to BTV, BLV, and BoHV-1 testing. Not all the samples were applicable to serological tests for OvHV-2 and BoHV-4 antigens (Table 2). Some sera showed cytotoxicity (indicated by cell death, probably caused by the sub-optimal condition of the samples) or they were not tested for insufficient serum quantity, and therefore, all these samples (n=64 for BoHV-4 and n=118 for OvHV-2) were excluded.

None of the tested animals resulted serologically positive for BLV,

Flock No.	Prefecture	SN titre							Toxic	NE	Total /flock
		Negative	4	8	16	32	64	128	256			
1	Hokkaido	12	1	3	1	0	0	1	0	2	0	20
2	Hokkaido	3	0	2	1	3	1	1	0	6	3	20
3	Hokkaido	4	1	2	0	0	2	1	1	9	0	20
4	Hokkaido	0	0	0	0	0	0	0	0	0	20	20
5	Hokkaido	14	0	0	1	0	1	1	0	3	0	20
6	Hokkaido	11	0	0	0	1	0	1	0	5	2	20
7	Hokkaido	11	0	0	2	0	0	0	0	7	0	20
8	Hokkaido	15	0	0	0	0	0	0	0	4	1	20
9	Hokkaido	6	0	3	1	0	0	0	0	9	1	20
9	Hokkaido*	1	0	0	0	0	1	1	0	2	0	5
10	Hokkaido	1	0	0	0	1	0	0	0	0	9	11
11	Iwate	8	0	0	0	0	1	0	0	11	0	20
12	Iwate	2	0	2	1	1	1	5	2	5	1	20
13	Aomori	7	0	0	0	1	1	4	0	6	1	20
14	Aomori	1	0	0	0	1	1	1	1	11	0	16
Total		96	2	12	7	8	9	16	4	80	38	272
Total seropositives				58 (37.66%**)								

NE: not executed due to insufficient aliquots for testing

*: goats

**: percentage computed excluding samples resulting toxic or not tested for insufficient serum quantity

Table 3. Results of serum neutralization (SN) screening test for antibodies to OvHV-2 virus in sheep from prefectures of northern Japan.

BoHV-1 and BoHV-4 antigens. Antibodies against BTV have been detected in 3 samples (1.11%), in two flocks (number 1 and 10) from Hokkaido, with a positivity rate at flock level of 10% and 9.09%, respectively.

The SN test revealed 58 samples, out of the 154 sera examined, positive for anti- OvHV-2 immunoglobulins (Tables 2 and 3); this corresponds to a seroprevalence of 37.66%. The overall flock seroprevalence was based on a single positive result within a flock with 85.71% of flocks being positive. The average incidence of seropositive animals in individual herds was from 11.11% (flock No.11) up to 85.71% (flock No.12). Titers ranged from 1:4 to 1:256 (Table 3). OvHV-2 infection was detected in 12 flocks out of the 14 sampled flocks. Levels of infection were found in flocks from Hokkaido, Aomori and Iwate prefectures. Hokkaido prefecture, the most involved in sheep production, had an overall seroprevalence of 30.97%. The serological results shows 80% of flocks were seropositive. Relatively higher prevalences were reported from the other two prefectures of Iwate and Aomori, 56.52% and 55.55%, respectively, with all the sampled flocks showing seropositive animals. Four rams and 54 ewes, and two goats were affected. Seroconversions were present in animals of all age categories from one year of age and older than eight years of age. Comparison between different age categories for the percentage of sheep positive for antibodies to OvHV-2 is reported in Table 4. Evaluation of the possible impact of OvHV-2 infection on production in the sampled flocks did not reveal a clear correlation with the reported levels of seropositive animals (Table 5).

Age category (year)	OvHV-2
1	37.5
2	44.44
3	65
4	20
5	54.16
6	21.42
7	30
≥8	33.33
Not known	34.78

Table 4. Comparison between different age categories for the percentage of sheep positive for antibodies to OvHV-2. Percentage computed excluding samples resulting toxic or not tested for insufficient serum quantity.

Discussion

This survey has demonstrated absence of positiveness for antibodies to BLV, IBR and BoHV-4 in sheep flocks in the northern prefectures of Japan, where the majority of the Japanese sheep are bred. The potential for infection in sheep remains consistent when considering that these infections are present in cattle [3,4,5,8]. In particular IBR is most frequent in Hokkaido, as indicated by reports from 2005 to 2011, with up to 42 outbreaks in 2009 [1]. Nevertheless, the importance of sheep in the epidemiology of IBR remains limited, considering the lower capacity of spreading the virus [18]. Similarly, sheep can be infected by BLV only via iatrogenic accident. However, according to the World Animal Health Organization (OIE), IBR and BLV are included in the list of reportable diseases of importance to international trade [19].

The screening for antibodies against BTV showed a very low prevalence in sheep from Hokkaido. However, the c-ELISA used for testing ensured specificity of the results and avoided non specific reactions due to cross reactivity with the Ibaraki virus, endemic in Japan [2]. This is the first serological evidence of BTV in sheep in the northern regions of Japan, as previous reports referred to central prefecture of Tochigi in the Honshu Island [1,2]. Giving that this seropositivity was observed in sera collected in 2007 and 2008, it might be in relation to the circulation of the virus occurred during the last reported outbreaks in cattle in 2005 and 2006 [1].

From these serological data it is clear that exposure to OvHV-2 is widespread across northern Japan with all the three prefectures having seropositive flocks. The numbers of samples tested from the prefectures varied, but the rate of positivity did not appear to relate directly to the number of samples tested. This survey provides the first serological evidence of the occurrence of the infection in sheep in the Iwate and Aomori prefectures. Interviews to farmers revealed that no previous investigations on the pathogen have been carried out in all of the randomly selected sampling units for this study, thus providing preliminary information on their epidemiology and distribution referring to years 2007 and 2008.

The seroprevalence reported in the present study (37.66%) was lower when compared with the previous study conducted in Japan (64.3%) [16]. In the U.S.A., OvHV-2 was associated with 53-59% of serologically positive sheep [20], in comparison with Germany where there have been reported prevalence of 75% [21], whereas in the

Flock No.	Prefecture	OvHV-2	Annual lambing rate	Annual lamb mortality rate	Annual culling rate	Mortality rate among adults
1	Hokkaido	33.33	NR	NR	NR	5
2	Hokkaido	72.72	0.72	1.29	0	4.76
3	Hokkaido	63.63	1.62	3.46	14.77	9.2
4	Hokkaido	NE	1.1	12.78	2.97	0
5	Hokkaido	17.64	1.61	20	11.73	8.33
6	Hokkaido	15.38	1.48	17.09	10.33	9.09
7	Hokkaido	15.38	1.58	16.92	6.66	2.22
8	Hokkaido	0	2.44	20.53	NR	NR
9	Hokkaido	40	1.23	0	0	0
10	Hokkaido	50	NR	NR	NR	10
11	Iwate	11.11	1.61	6.89	0	11.76
12	Iwate	85.71	1.38	9.83	24.03	4.8
13	Aomori	46.15	1.54	25.35	17.64	2.94
14	Aomori	80	1.14	21.87	0	9.09

NE: not executed due to insufficient aliquots for testing

NR: not recorded

Table 5. Comparison between different production parameters for the percentage of sheep positive for antibodies to OvHV-2.

Middle East even above 70% to 95% [22]. Furthermore, generally, in contrast to a lower degree of seropositivity in MCF-susceptible species, including cattle, bison, deer, caribou, elk (*Cervus elaphus*) and moose, which ranges from a few percent to 50% seropositive [20,21], species that may harbour MCF viruses, including sheep and goats, have a high frequency of seropositivity (>90%) [20], indicating their status as inapparent carriers of MCF viruses. The reported lower prevalence might be due to the use of SN test, different from the tests used in other studies. For example, complement fixation test was used in the previous study conducted in Japan [16]. In the U.S.A. and Germany, OvHV-2 antibodies were detected using enzyme-linked immonosorbent assay [20,21], and in Israel was used PCR [22]. Nevertheless, ideally a comparison with other tests would increase information about the reliability of infection rate obtained with the test used but was outside the scope of this study.

Although no diagnostic measures were in place, evaluation of the possible impact of MCF infection on production in the sampled flocks was considered taking into account that sheep can develop a mild form of MCF [12,13], with an eventual reduced productivity. This study did not reveal any clear correlation between production and the reported levels of seropositive animals. This is in line with the general subclinical course of the infection in MCF reservoir host species.

In this study, considering that one sheep flock (Hokkaido 9) was breed with a large number of goats, additional goat samples have been collected for testing MFC virus infection. Two caprine serum samples resulted positives. Reasonably, immune reaction reported in goats could refer to CpHV-2, genetically related, and cross reacting with the other MCF herpes viruses. However, seropositivity could be ascribable to immune reaction against OvHV-2, taking into account the close contact with infected sheep (40% resulted positive in the sheep flock) and the susceptibility of goats to the ovine virus. OvHV-2-specific DNA sequences have been reported in goats [23]. In addition, OvHV-2 sequences could be detected by PCR in 17% of goats surveyed in Indonesia [24]. Discrimination between CpHV-2 and OvHV-2 was not possible by the used serological test.

Considering the potential direct or indirect adverse effects on bovine welfare and production, suitable intervention and control measures should be introduced to avoid diffusion and impact on valuable breeding cattle farming. In some farms, other domestic animals and in particular cows were housed close to sheep pens or had access to common pastures. A sheep flock (Hokkaido 7) originated from a farm mainly focused on dairy cattle breeding, thus being in close contact with a herd of 700 Japanese black cows. Similarly, preventive measures should be considered to protect wild fauna. Cervids, such as sika deer which in Japan is represented by a large population, are even more sensible than cattle to OvHV-2 infection. Many deer die within 48 h of the first clinical signs and affected bison generally die within three days [25].

Primarily, close contacts with sheep flocks should be avoided. However, although most infections occur when the carrier host and susceptible animals are in close contact, transmission of OvHV-2 has been reported when cattle was separated by a distance of at least 70 meters from lambs [10]. Similarly, SA-MCF was also reported in bison herds up to 5 km from a lamb feedlot [10].

An important aspect to be considered is that MCF-associated OvHV-2, shed intermittently in nasal secretions [26], appears to be transmitted by contact or aerosol, mainly from lambs under one year of age, particularly by six to nine months old lambs [27]. Taking into account that the majority of lambs are not infected until after two months of age, under natural conditions [27], production of virus-free hosts may also be considered. If lambs are removed from contact with infected sheep prior to that age, by early weaning and isolation, they remain uninfected and can be raised virus free [28]. This knowledge is being used by farmers to produce OvHV-2-free sheep in USA and Europe [29]. Similarly, the production of CpHV-2-free goats could be obtained.

The demonstration of OvHV-2 circulation in sheep flocks in the northern prefectures of Japan, based on serological analysis, advanced the knowledge on pathogens affecting domestic sheep in Japan. These interesting findings deserve further evaluations in order to examine the full extent of the problem in small ruminant populations. Knowledge and awareness on the disease should be improved and disseminated to veterinarians and farmers.

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References

1. World Organisation for Animal Health (Office International des Épizooties: OIE) (2012) World Animal Health Information System (http://www.oie.int/wahis/public.php accessed on 25 December 2012).
2. Shimizu S, Toyota I, Arishima T, Goto Y (2004) Frequency of serological cross-reactions between Ibaraki and bluetongue viruses using the agar gel immunodiffusion test. Vet Ital 40: 583-586.
3. Ogawa T, Ishikawa M, Inoue T (1995) Trends of epidemics of bovine infectious diseases in Japan during 1987-1993. JARQ 29: 263-267.
4. Ogino H, Inui S, Narita M (1996) Demonstration of infectious bovine rhinotracheitis virus antigen by immunoperoxidase method in tissues of aborted bovine fetuses preserved for 25 years in paraffin blocks. J Vet Med Sci 58: 459-460.
5. Ogino H, Kaneko K, Nakabayashi D, Watanabe T, Murayama J (1996) Pathology of bovine abortion and newborn calf death caused by dual infection with Chlamydia psittaci and infectious bovine rhinotracheitis virus. J Vet Med Sci 58: 67-70.
6. Ackermann M (2005) Virus in sheep's skin. Schweiz Arch Tierheilkd 147: 155-164.
7. Ackermann M (2006) Pathogenesis of gammaherpesvirus infections. Vet Microbiol 113: 211-222.
8. Masayuki Y, Yoshihisa S, Michiru I, Noriko A, Kunitoshi I (2005) Infiltration of Bovine Herpesvirus 4 into the Hokkaido District, Japan. Journal of the Japan Veterinary Medical Association 58: 33-36.
9. Whetstone CA, Evermann JF (1988) Characterization of bovine herpesviruses isolated from six sheep and four goats by restriction endonuclease analysis and radioimmunoprecipitation. Am J Vet Res 49: 781-785.
10. World Organisation for Animal Health (Office International des Épizooties: OIE) (2009) Technical disease cards, OIE, Paris, Malignant catarrhal fever, p. 2.
11. Bublot M, Wellemans G, Van Bressem MF, Dubuisson J, Pastoret PP, et al. (1991) Genomic diversity among bovine herpesvirus 4 field isolates. Arch Virol 116: 1-18.
12. Buxton D, Reid HW, Finlayson J, Pow I, Berrie E (1985) Transmission of a malignant catarrhal fever-like syndrome to sheep: preliminary experiments. Res Vet Sci 38: 22-29.
13. Li H, O'Toole D, Kim O, Oaks JL, Crawford TB (2005) Malignant catarrhal fever-like disease in sheep after intranasal inoculation with ovine herpesvirus-2. J Vet Diagn Invest 17: 171-175.
14. Li H, Keller J, Knowles DP, Crawford TB (2001) Recognition of another member of the malignant catarrhal fever virus group: an endemic gammaherpesvirus in domestic goats. J Gen Virol 82: 227-232.

15. World Organisation for Animal Health (Office International des Épizooties: OIE) (2012) Terrestrial Manual, OIE, Paris, Enzootic bovine leukosis, Chapter 2.4.11. p. 9-10.

16. Sentsui H, Nishimori T, Nagai I, Nishioka N (1996) Detection of sheep-associated malignant catarrhal fever virus antibodies by complement fixation tests. J Vet Med Sci 58: 1-5.

17. Lelli R, Portanti O, Langella V, Luciani M, Di Emidio B, et al. (2003) Production of a competitive ELISA kit for the serological diagnosis of bluetongue disease. Vet Ital 47: 5-12.

18. Galais-Duhamel C (2006) Les Herpesvirus bovins chez les ruminants. Ph.D. thesis, National veterinary school of Alfort, France.

19. World Organisation for Animal Health (Office International des Épizooties: OIE) (2012) OIE listed diseases 2012 (http://www.oie.int/en/animal-health-in-the-world accessed on 23 December 2012).

20. Li H, Shen DT, Jessup DA, Knowles DP, Gorham JR, et al. (1996) Prevalence of antibody to malignant catarrhal fever virus in wild and domestic ruminants by competitive-inhibition ELISA. J Wildl Dis 32: 437-443.

21. Frölich K, Li H, Müller-Doblies U (1998) Serosurvey for antibodies to malignant catarrhal fever-associated viruses in free-living and captive cervids in Germany. J Wildl Dis 34: 777-782.

22. Brenner J, Perl S, Lahav D, Garazi S, Oved Z, et al. (2002) An unusual outbreak of malignant catarrhal fever in a beef herd in Israel. J Vet Med B Infect Dis Vet Public Health 49: 304-307.

23. Wiyono A, Baxter SI, Saepulloh M, Damayanti R, Daniels P, et al. (1994) PCR detection of ovine herpesvirus-2 DNA in Indonesian ruminants--normal sheep and clinical cases of malignant catarrhal fever. Vet Microbiol 42: 45-52.

24. Wiyono A (1999) The detection of ovine herpesvirus 2 in reservoir host of malignant catarrhal fever in Indonesia by means of polymerase chain reaction. J Ilmu Ternak dan Vet 4: 121-127.

25. O'Toole D, Li H, Sourk C, Montgomery DL, Crawford TB (2002) Malignant catarrhal fever in a bison (Bison bison) feedlot, 1993-2000. J Vet Diagn Invest 14: 183-193.

26. Taus NS, Traul DL, Oaks JL, Crawford TB, Lewis GS, et al. (2005) Experimental infection of sheep with ovine herpesvirus 2 via aerosolization of nasal secretions. J Gen Virol 86: 575-579.

27. Li H, Snowder G, O'Toole D, Crawford TB (1998) Transmission of ovine herpesvirus 2 in lambs. J Clin Microbiol 36: 223-226.

28. Li H, Snowder G, Crawford TB (1999) Production of malignant catarrhal fever virus-free sheep. Vet Microbiol 65: 167-172.

29. Müller-Doblies UU, Egli J, Li H, Braun U, Ackermann M (2001) [Malignant catarrhal fever in Switzerland. 1.Epidemiology]. Schweiz Arch Tierheilkd 143: 173-183.

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Short communication

Numerical taxonomy of the genus *Pestivirus*: New software for genotyping based on the palindromic nucleotide substitutions method

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A B S T R A C T

The genus *Pestivirus* from the family *Flaviviridae* is represented by four established species; Bovine viral diarrhea virus 1 (BVDV-1); Bovine viral diarrhea virus 2 (BVDV-2); Border disease virus (BDV); and Classical swine fever virus (CSFV); as well a tentative species from a Giraffe. The palindromic nucleotide substitutions (PNS) in the 5′ untranslated region (UTR) of *Pestivirus* RNA has been described as a new, simple and practical method for genotyping. New software is described, also named PNS, that was prepared specifically for this PNS genotyping procedure. *Pestivirus* identification using PNS was evaluated on five hundred and forty-three sequences at genus, species and genotype level using this software. The software is freely available at www.pns-software.com.

The genus *Pestivirus* from the family *Flaviviridae* is represented by four established species; *Bovine viral diarrhea virus 1* (BVDV-1), *Bovine viral diarrhea virus 2* (BVDV-2), *Border disease virus* (BDV) and *Classical swine fever virus* (CSFV) as well as a tentative species from a "Giraffe" (King *et al.*, 2012). The *Pestivirus*, is single-stranded, has positive polarity RNA and has a sequence of about 12,500 nucleotides. It can be divided into three regions: a 5′-untranslated region (UTR), a single large open reading frame encoding region, and a 3′-UTR. The 5′-UTR is highly conserved among all members within the genus *Pestivirus*, thus it is useful for the characterization of species or genotypes. The primary structure analysis, by sequence alignment and construction of phylogenetic trees, is the most common method for the classification of *Pestivirus* strains. Genetyx-Mac, DNASIS, Clustal X (Thompson et al., 1997) are among the software used for typing virus strains based on sequence alignment. In reality, the genomic sequence is tri-dimensional. The reproduction of the third structure is highly problematic. However, it is relatively easy to predict the secondary structure, according to the most probable nucleotide binding, with lowest folding energies. The secondary structure of the 5′-UTR can be divided into four domains, A–D, with domain D encompassing the 3′ two thirds of the UTR predicted to fold into a complex palindromic stem–loop structure, including an internal ribosome entry site (IRES), as observed in poliovirus (Deng and Brock, 1993; Harasawa, 1994). This therefore represents critical regions of the 5′-UTR, responsible for translational, transcriptional and replicational events in pestiviruses.

The *Pestivirus* genome has a relatively long 5′-UTR upstream of the polyprotein open reading frame. Although the nucleotide sequence of the 5′-UTR is well conserved among the members of the *Pestivirus* genus, the 5′-UTR has been shown to contain at least three variable loci. The nucleotide substitutions in these variable loci are of particular importance because the 5′-UTR of the positive-sense RNA viruses generally includes regulatory motifs, which are indispensable for viral survival. Therefore, random mutations at the 5′-UTR have a high probability of incompatibility with viral survival. Thus stable nucleotides at the 5′-UTR level are assumed to be important in terms of virus evolutionary history.

Nucleotide sequences at the three variable loci, V1, V2 and V3, in the 5′-UTR of pestiviruses have been shown to be palindromic and capable of forming a stable stem–loop structure peculiar to each *Pestivirus* species. Nucleotide substitutions in the stem regions always occur to maintain the palindromic sequence and thereby form this stable stem–loop structure. Thus, this type of mutation was referred to as "palindromic nucleotide substitutions" (PNS). Based on this observation of nucleotide variations among virus strains in the three specific palindromes of the 5′-UTR, a method of genotyping has been developed (Harasawa and Giangaspero, 1998). The method named palindromic nucleotide substitutions (PNS) appeared to be simple and practical, showing comparable results with other procedures based on the primary structure comparison.

According to palindromic nucleotide substitutions, 543 sequences (Table 1) have been segregated into nine species within

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Table 1
Summary of *Pestivirus* strains (n 543) evaluated according to the Palindromic nucleotide substitution (PNS) method at the 5′ untranslated region of RNA.

Species	Number of strains	Host	Geographical origin
BVDV-1	281	Cattle, Sheep, Pig, Deer, Roe deer, Human, Contaminant	Argentina, Austria, Belgium, Brazil, Canada, China, Belgium, France, Germany, India, Ireland, Italy, Japan, New Zealand, Slovakia, South Africa, Spain, Sweden, Switzerland, UK, USA
BVDV-2	77	Cattle, Sheep, Contaminant	Argentina, Austria, Belgium, Brazil, Canada, France, Germany, Italy, Japan, Netherland, New Zealand, Slovakia, Tunisia, UK, USA
BVDV-3[a]	3	Cattle	Brazil, Thailand
BDV	131	Sheep, Pyrenean chamois, Cattle, Pig, Reindeer, Wisent	Australia, France, Germany, Japan, New Zealand, Spain, Switzerland, Tunisia, Turkey, UK, USA
BDV-2[a]	5	Sheep, Goat	Italy
CSFV	43	Pig, Sheep	China, France, Garmany, Honduras, Italy, Japan, Malaysia, Netherlands, Poland, Russia, Spain, Switzerland, USA
Pronghorn[a]	1	Pronghorn	USA
Giraffe[a]	1	Giraffe	Kenya
Bungowannah[a]	1	Pig	Australia

[a] Tentative species.

the genus *Pestivirus* (Giangaspero and Harasawa, 2011). In addition to the four established species, Harasawa et al. (2000) characterized the taxonomic status of the novel giraffe strain, based on the 5′ untranslated region, as a new cluster among *Pestivirus* species. Furthermore, four other tentative species (BVDV-3, BDV-2, Pronghorn and Bungowannah) have been recently proposed (Vilček et al., 2005; Kirkland et al., 2007; Giangaspero and Harasawa, 2011). Genotypes have been identified in species showing heterogeneity: *Bovine viral diarrhea virus 1* (Giangaspero et al., 2001), *Bovine viral diarrhea virus 2* (Giangaspero et al., 2008; Giangaspero and Harasawa, 2011), *Border disease virus* (Giangaspero, 2011) and *Classical swine fever virus* (Giangaspero and Harasawa, 2008). The observation made on the nucleotide sequences of the three variable loci at the level of the 5′-UTR genomic region of *Pestivirus* strains allowed identification of consensus motifs shared by all the *Pestivirus* species. The characteristic palindromic nucleotide substitutions have been identified at genus, species and genotype level, respectively (Tables 2 and 3). The palindromic loci represented, with about 80 nucleotides, a very limited portion of the virus genome. Within these short sequences, only 21 nucleotides were required to characterize to the genus level with a degree of certainty. Species were determined by the evaluation of only 6–19 nucleotides. Similarly, the genotype was defined with only 6–10 nucleotides. These specific viral properties showed the high specificity of the PNS method and the reliability of the results.

The main limitation of this method is the manual searching of relevant base pairings and direct observation of the sequence. The aim was to improve the PNS method by computerising the procedure, so specific software was designed to facilitate easy use and rapid testing with consistent and reliable results for *Pestivirus* identification.

The program was developed using the "C++" programming language (Ellis and Stroustrup, 1990) and adapted to run under the Windows operating system. The software was constructed with an ability to evaluate up to 15,000 bases of virus nucleotide sequences. Generally, RNA virus sequences from the 5′-UTR genomic region, that are deposited in international databases include approximately 250–350 nucleotides. However, the software will be able to process RNA sequence data from a whole virus genome. The primary objective was to identify the three variable loci V1, V2 and V3 in the test sequence which is characteristic for this genotyping procedure. The sequences to be tested were prepared as a text file (.txt) for input into the program. No other characters than bases were allowed in the file.

Once the compatibility of the input file was verified, through the first stage of the process, the program loaded the sequence into its memory. The three variable sequences were then identified automatically by the program and the secondary structures were determined according to the Watson–Crick base pairings; with strong bindings as adenine uracil or cytosine guanine, tolerated pairings in secondary structure as guanine uracil and pairings without binding forming bulges as cytosine cytosine. Since timine is equivalent to uracil, the program was adapted to translate uracil as timine in case test sequences were constructed with uracil. In addition, the nucleotides were treated the same whether they were expressed in lower case or capital letters in the sequence file.

1. PNS class

The most important class of the software was named "PNS", in addition to a series of data structure definitions, it contains two fundamental methods which are respectively: the *SearchPNS*, for loading the test nucleotide sequence into its memory to identify the V1, V2 and V3 structures; the *CreateHTML*, which analysis the structures of the V1, V2 and V3 regions using known parameters to identify the PNS and also to create an html file containing the results of the analysis.

1.1. Uploading data and initial analysis for key data structures

The PNS software uses four data structures to memorise and keep data information available during the analytical process.

(1) All possible nucleotide pairings and the related bindings are memorised using a character matrix.
(2) The nucleotides read by the input file are stored in an array (Lodi and Pacini, 1998) with a maximum dimension of 15,000 bases.
(3) The analytical parameters for identifying the genus, species and genotypes are stored in three arrays of a structure (*struct*) named *Records*, which contains the following fields: *name*, *structure*, *position*, *left*, *right*, and *binding* respectively.
(4) The V1, V2 and V3 structures are stored using three pointers list (Lodi and Pacini, 1998) defined by two different type of *struct*. The first defined the single node in the list; the second defined the structure of the list type. Nodes were linked in a sequential order by virtual bindings represented by pointers, addresses of memory, so that a single node contains the pointer at a following node. The list node contains the following fields: *left*, *right*, *right_2*, *binding_type*, *prior*, *next*. A schematic presentation of the list management in the program memory is described in Fig. 1.

1.2. PSet abstract structure

On the base of the model proposed by Apicella (1999), an abstract parametric class or template, called *PSet*, was determined

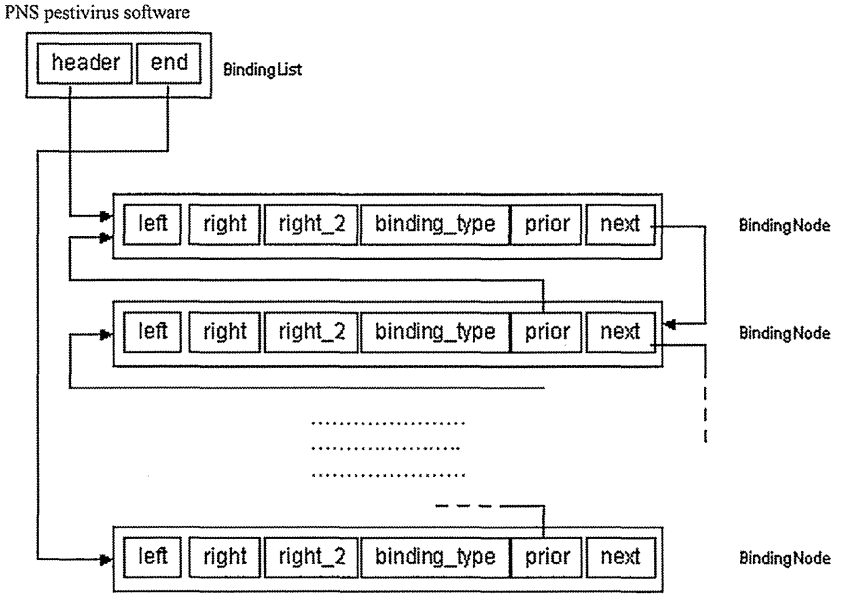


Fig. 1. Schematic presentation of the pointer list management in the PNS program memory.

to obtain a dynamic and flexible structure allowing the management of a double linked pointer list of generic type T objects.

The use of *PSet* was necessary for the program, expecially during the phase of V1, V2 and V3 structure determination where the selection of the correct structure depended on the base of defined criteria among a large number of possible structures. A *PSet* class facilitated the entire list recording, evaluating all the possible structures and identifying the correct one.

1.3. Construction of the V1, V2 and V3 palindromic structures

The appropriate structure of type V1, V2 or V3 from a tested sequence was selected from a large number of possible structures and identified according to well defined criteria, following the specific program procedures. The three variable loci in the test sequence were identified starting with the V2 locus due to the invariable number of nucleotides that exist in the palindrome (23 bases); and the characteristic conserved 4 guanine sequences of the loop (5′-GGGG-3′). The calculation of the secondary structure was based on a search of palindromes with the highest number of Watson-Crick base pairings with strong bindings (C-G; G-C; A-U; U-A), which are most probable, with the priority for the stable structures in confront to tolerated pairings (G*U; U*G) and pairings forming bulges (CC; AA; AC; AG; CA; GG; GA; UU; CU; UC). The construction of the palindrome structures respected the principle of the correct curbing of the sequence at the loop level, thus avoiding formation of base pairing generating strong or weak, tolerated bindings. To clarify, if a loop is present with 3 nucleotides n_1, n_2, n_3 the potential binding occurring between n_1 and n_3, for example G–C, was not considered. Similarly, in cases with 4 nucleotides (n_1, n_2, n_3) the potential binding between n_1 and n_4 and between n_2 and n_3 were not considered. The first step carried out by the software is the verification of the presence of a structure compatible with the V2 locus in a test sequence, giving that the determination of the following structures depended exclusively on this component. In case no V2 structure could be identified, the analyzed sequence had to be considered as not corresponding to 5′-UTR of the genus *Pestivirus*, resulting in the termination of the program. Alternatively, if one or more V2 structures are identified in a given sequence, the program selected the appropriate one and continued with the analysis.

The next palindrome to be identified was the V3 locus, according to the conserved separation by generally 3 nucleotides, but in some cases just 2, as determined for strains VM, CP1874 and Marloie, from the V2 locus end and the starting of the V3 sequence. Consequently, the identification of the V3 was only possible when the V2 locus was identified. Similarly for V2, the construction was based on the search of the secondary structure with the highest number of Watson–Crick base pairings with strong bindings, taking into account the approximate length of the sequence, which is variable from 15 to 20 nucleotides, with a 6–7 base pairing composed stem and a 3–5 nucleotide loop. The construction of the stable palindrome was more problematic due to the variability of the sequence length. The V3 starting position was the third or fourth nucleotide located after the end of the V2 locus sequence. As a first step, the possible V3 palindromes were identified starting from the fourth nucleotide after the V2 end. From this nucleotide, n possible V3 structures named $v_{i=1,\ldots,n}$, were determined. For each v_i structure was identified the one with the highest value of strong bindings. In the case of two or more v_i having the same maximum value, the selection was done on the lowest index i among them, giving that the structure showed the fewest nucleotides and are therefore more stable. At the second stage, the procedure was repeated starting from the third nucleotide after the V2 end identifying possible V3 structures identified as $v_{j=1\ldots,m}$. At the end of the two stages, the respective resulting structures v_i and v_j were compared to determine the correct V3. The structure showing the highest number of strong bindings and the minimum dimension (constructed starting from the fourth nucleotide after V2, irrespective of the number of nucleotides, in case of similarity) was selected as V3 sequence, resulting in the output produced by the program.

The V1 was the last identified palindrome with the appropriate construction with a stem, composed by the highest number of strong bindings, interrupted by a lateral palindrome and the characteristic CC bulge and ending with a variable loop. Particular aspects had to be taken into account, as the variable number of nucleotides included in the palindrome, mainly 39 and up to 42 in BVDV 2 and Giraffe, and the variable starting point of the V1 in the genomic sequence, depending on the type of primers used for RT-PCR reaction. The parameters for the determination of the V1 locus were related to the position of the V2 locus in the sequence, as per V3.

Table 2
Palindromic nucleotide substitutions (PNS) characteristic to the genus *Pestivirus*. The position of base pairings is defined by numbering from the bottom of the variable locus.

	Locus	Characteristic PNS markers
Genus		
	V1	Absence in position 22 – size of V1 21 bp (exception U); C C bulge in position 11; A-U in position 10; C-G in position 8 (exceptions U*G, U-A and G G bulge); U-A in position 7 (exception G-C and A A bulge); A in position 6 (exception G); U*G in position 5; U in position 5 right nucleotide; G-C in position 4
	V2	GGGGU loop (exception GGGGC); C-G in position 8 (exception U*G).
Species		
BVDV-1	V1	U-A in position 15 (exception U*G or C-G);
	V2	G-C in position 5 (exception A-U);
	V3	G-C in position 5; A in position 10 (exceptions A-U, G-C or A C, A A or G A bulges or absence).
BVDV-2	V1	A-U or A C bulge in position 20 (exceptions G*U, C C or A A bulges); A,G or U in position 21 (exception G G);
	V2	U-A or U*G in position 6 (exception C A bulge);
	V3	A-U or A C bulge in position 7 (exception G-C).
BVDV-3 tentative species (HoBi group)	V1	U-A in position 15;
	V3	G-C or G*U in position 3; A-U or G-C in position 7; A in position 10
BDV	V1	G-C or A-U in position 15 (exceptions C U and A C bulges);
	V3	U C and U U bulges or U*G in position 7 (exceptions A-U, U-A and C C, A C, C U and C A bulges).
BDV-2 tentative species (Italian ovine isolates)	V1	U-A or C A bulge in position 15;
	V3	G*U or G G bulge in position 8
CSFV	V1	U-A in position 13 (exception U*G);
	V3	U-A in position 2; U or C in position 8 (exception A).
Pronghorn tentative species	V1	G-C in position 2; U-A in position 9; U-A in position 12; U-A in position 15;
	V2	G-C in position 4;
	V3	G A bulge in position 5.
Giraffe tentative species	V1	C-G in position 2; U*G in position 20;
	V2	C-G in position 7;
	V3	C-G in position 4; G*U in position 7.
Bungowannah tentative species	V1	A-U in position 2; G-C in position 7; U-A in position 9; U-A in position 12; G-C in position 13;
	V2	A-U in position 3; G-C in position 4;
	V3	U-A in position 4; G A bulge in position 10; A in position 11.

Starting from the beginning of the V2 locus and proceeding back along the sequence, for a minimum of ten nucleotides, G or A's are identified that are followed by a nucleotide and then by a U and C (the description is following a backward search, in the sequence this appeared as 5′-nnnnCUnG/Ann.nnV2-3′). All the CUnG/A sequences are retained and recorded in a list. In case no CUnG/A sequences are identified, the program finished the procedure for the construction of the V1 locus. Furthermore, continuing backward, all the sequences C and A, followed by a nucleotide and a C or A or G (5′-n.nG/A/CnACnn.nnCUnG/An.nV2-3′) are identified and recorded in a list. This search is related to the definition of the core of the V1 locus, identifying the two C's that comprise the characteristic bulge located on the stem of the palindrome and it

Table 3
Palindromic nucleotide substitutions (PNS) characteristic to the *Pestivirus* species genotypes. The position of base pairings is defined by numbering from the bottom of the variable locus.

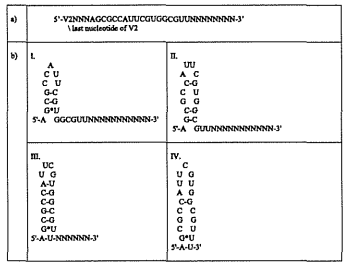
	Locus	Characteristic PNS markers
BVDV-1 genotypes		
BVDV-1a	V1	U*G or C-G in position 14.
	V2	G*U or G-C in position 7.
	V3	A-U in position 4.
BVDV-1b1	V1	U-A in position 14 (exception G A bulge).
	V2	A-U in position 7 (exception A C bulge).
	V3	G-C in position 4 (exception A-U).
BVDV-1b2	V1	U-A in position 14 (exception A A bulge).
	V2	G*U or G-C in position 7.
	V3	G-C in position 4.
BVDV-1c	V1	C-G in position 14 (exception C A bulge).
	V2	A C bulge in position 7.
	V3	A-U in position 4.
BVDV-1d	V1	C-G in position 14.
	V2	A-U in position 7.
	V3	G-C in position 4.
BVDV-1e	V1	C-G in position 14.
	V2	G-C in position 7.
	V3	A C bulge in position 2; G-C in position 4.
BVDV-1f	V1	C-G in position 14.
	V2	A-U in position 7.
	V3	A-U in position 4.
BVDV-1g	V1	C-G in position 14; U*G in position 17.
	V2	G-C in position 7.
	V3	G*U in position 4.
BVDV-1h	V1	C-G in position 14.
	V2	C U bulge in position 6; G-C in position 7.
	V3	G-C in position 4.
BVDV-1i	V1	C-G in position 14.
	V2	G*U in position 1; G-C in position 7.
	V3	G-C in position 4; A A or G A bulges in position 8.
BVDV-1j	V1	C-G in position 14.
	V2	A-U in position 1; G- C in position 7; A C bulge in position 9.
	V3	G-C in position 4; G-C or G A bulge in position 6.
BVDV-1k	V1	C-G in position 14; C-G or C A bulge in position 16; A A bulge in position 19.
	V2	A-U in position 1; G-C in position 7.
	V3	G-C in position 4.
BVDV-1l	V1	CG in position 14.
	V2	UA in position 1; AC in position 7.
	V3	GC in position 4.
BVDV-1m	V1	C-G in position 14.
	V2	G-C in position 7; A C bulge in position 9.
	V3	G-C in position 4; A A bulge in position 6.
BVDV-1n	V1	C-G in position 14; C-G in position 15.
	V2	G-C in position 7; A-U in position 5.
	V3	G-C in position 4.
BVDV-1o	V1	C-G in position 14.
	V2	G-C in position 7; U-A in position 5.
	V3	G-C in position 4.
BVDV-2 genotypes		
BVDV-2a	V1	C-G in position 16; U*G, C-G or U-A in position 18.
BVDV-2b	V1	G-C in position 12; U-A in position 16; G A, G G or A C bulges in position 17; G G or G A bulges in position 18.
	V3	Higher V3 loop, U in position 10.
BVDV-2c	V1	G-C in position 12; C-G in position 14; C-G in position 16; G A, G G or A C bulges in position 17; G A bulge in position 18.

Table 3 (*Continued*)

	Locus	Characteristic PNS markers
	V3	Higher V3 loop, C in position 10.
BVDV-2d	V1	A-U in position 9; A-U in position 12; U-A in position 16; G A, G G or A C bulges in position 17; G A bulge in position 18.
	V3	Higher V3 loop, U in position 10.
BVDV-2e	V1	U-A in position 8; C C bulge in position 20.
	V3	G-C in position 7.
BDV genotypes		
BDV-a	V1	A-U or C U bulge in position 9; A A or A G bulges in position 18 (exception G G bulge).
	V2	A-U in position 1.
	V3	A A, G A or A C bulges in position 8.
BDV-b	V1	G-C in position 9; G-C or G G bulge in position 18; G*U or G G bulge in position 20.
	V2	G*U in position 1.
	V3	U-A or C A bulge in position 8.
BDV-c	V1	G-C or U C bulge in position 20; U or U U bulge in position 21.
	V2	A C bulge in position 1.
	V3	C C bulge in position 7.
BDV-d	V1	G-C or A-U in position 9; U*G, G-C, G*U or G G bulge in position 18 (exception A G bulge).
	V2	G*U, G-C in position 1 (exceptions A-U and C U bulge).
	V3	U-A, C-G, U*G, A A or C A bulges in position 8.
BDV-e	V1	U-A, C-G or U*G in position 16.
	V3	C U bulge in position 1; G*U or U U bulge in position 2.
BDV-f	V3	U-A in position 2; U*G in position 7; U or C in position 8.
BDV-g	V1	G-C in position 3; U-A or C-G in position 16.
	V3	G-C in position 4.
BDV-h	V2	G-C in position 5.
	V3	G-C or A C bulge in position 2; C-G in position 7; U U bulge in position 9; U Uor C U bulge in position 10.
CSFV genotypes		
CSFV-a	V1	A C bulge in position 15 (exception G-C).
	V2	U-A in position 5; G:Y in position 7.
	V3	A-U in position 1.
CSFV-b	V1	G-C in position 15; A G bulge in position 19; U-A in position 20.
	V2	A-U in position 5; A C bulge in position 7.
	V3	A-U in position 1; U C bulge in position 6.
CSFV-c	V1	A-U in position 15.
	V2	U-A in position 5; G-C in position 7.
	V3	A G bulge in position 1.

then considers two more nucleotides, one forward and one back (5′-n.n*nG/A/CnACnn.nnCUnG/An*n.nV2-3′). The V1 locus is completed after considering five nucleotides back, of which the first is usually U (5′-n.n*nnnnUnG/A/CnACnn. . .nnCUnG/An*n.nV2-3′) and forward height nucleotides, of which the second, the third and the fourth were characteristic AUG (except for the BVDV-1b strain Sanders where A is changed with G) (5′-n.n*nnnnUnG/A/CnACnn.nnCUnG/AnnAUGnnnn*n.nnV2-3′). The remaining part of the stem with the lateral palindrome was also constructed. At the level of the lateral palindrome, the opposite was included as a deletion point in order to correctly equilibrate the stem. For each CUnG/A identified sequences was performed the construction of a palindromic locus according to each defined C/A/GnAC following sequences. All possible sequence structures

Table 4
Palindromic nucleotide substitutions (PNS) genotyping method for genus *Pestivirus*. Construction of the V3 palindrome locus with determination of the length of the sequence. The example was applied on the V3 locus of strain Lees (accession number U65051) which includes 18 nucleotides. Until now, observations indicated V3 to change form 15 to 20 nucleotides, however, it is not possible to exclude new and different data. (a) Identification of the linear sequence. The first nucleotide of V3 had a highly conserved position as the fourth nucleotide after the end of V2, and as the third nucleotide only in some strains. Due to the variability of the V3 sequence, the last nucleotide was not determined. (b) Determination of the most stable palindromic structure. The sequence was gradually replied onto itself, searching for a maximum number of strong bindings. I. A palindromic structure with 2 strong bindings and 1 weak binding was identified with a 13 nucleotide sequence. II. Sequence with 16 nucleotide showed 3 strong bindings. III. Sequence with 18 nucleotides showed 6 strong bindings and 1 weak binding. IV. A longer sequence showed instable structure. The 18 nucleotide sequence resulted compatible with V3 locus.



were constructed (30-n+1) and included in a list of candidate V1's in case the distance between the beginning of the sequence and the first element before a CUnG/A did not exceed the 30 nucleotides. V1 candidate structures were constructed and considered in the list according to the *m* number of C/A/GnAC identified sequences, with variable loop dimension not exceeding 30 nucleotides. The selected sequence showed the highest number of strong bindings in the stem, and in case of similarity, selected those with the fewer nucleotides. The absence of the sequence C/A/GnAC indicated the incompleteness of the V1 locus therefore incomplete palindromes were constructed identifying the starting point of the V1, at the possible level of nAC, AC or C from an incomplete C/A/GnAC sequence, and determining a series of possible V1 sequences, with different dimensions, with a variable loop starting from the first, second or third nucleotide of the tested sequence, included in the V1 candidate list (the V1 were constructed only with variable loop dimension that did not exceed 30 nucleotides).

2. Log file

The text file "*log.txt*" created during the execution of the program is important for monitoring its execution. Its content includes all the different processes required for the determination of the final result. In the case of an unclear or incoherent result, the analysis of this file allows for the cause of the problem to be displayed as a "malfunction" of the program.

2.1. Application of Pestivirus identification keys and html file output

After comparison of the identified structures in all genus, species and genotypes and taking into account all of the known exceptions for divergent base pairs in the genotypes, the final result is created and displayed as a .html file by the program. This document shows graphically the portions of the sequence reconstructed and identified as the palindromic V1, V2 and V3 variable loci. The corresponding PNS *Pestivirus* identification keys in the tested sequence are highlighted at the genus level and with the species specific PNS parameters indicating the matches with characteristic base pairings. At the genotype parameter level, the highlighting was applied

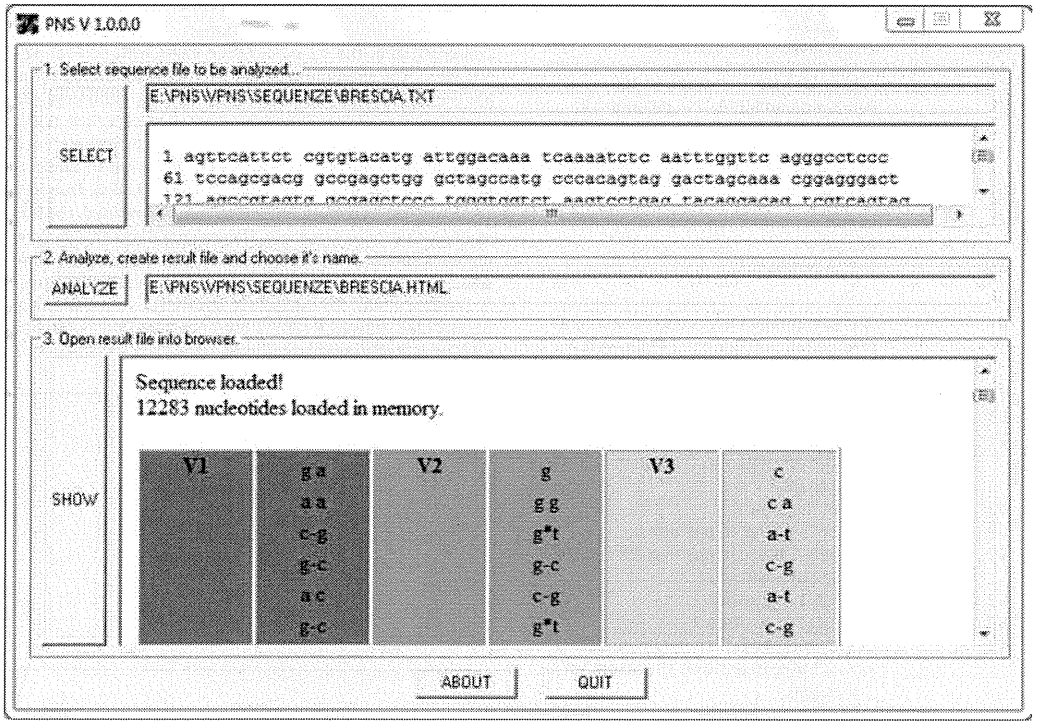


Fig. 2. PNS software allowed a simple and intuitive utilization, through selection of sequence to be tested included in a text file format, and display of secondary structure relevant loci with subsequent analytical classification.

only in relation to the identified species. For each PNS pattner, the related control result was included in the output file, showing the expected base pairing and the observed one at each position in the structures, to provide evidence of any relevant characteristic in the strategic region of the 5′-UTR.

The software was tested using the nucleotide sequences in the 5′-UTR region of 543 *Pestivirus* strains of the species BVDV-1, BVDV-2, BDV, CSFV, and of the tentative Giraffe species as well as BVDV-3, BDV-2, Pronghorn and Bungowannah. The sequences were from different geographical origins, from different host species and also from contaminants of biological products, and were obtained either from the GenBank DNA database, provided by authors, or obtained in our laboratories (Table 1) (detailed list of analysed strains available under request).

The PNS software developed here resulted in a satisfactory prototype, as demonstrated by the successful application and testing of a large number of virus strains. The sequences were correctly displayed with their palindromes and the application of the keys for *Pestivirus* identification showed clear results presented in the output file. The identification step allowed for three distinct evaluations. The first was the comparison with the genus specific PNS, identifying the appurtenance to the *Pestivirus* genus. Further evaluations only continued if PNS matches were obtained. Other comparisons were then performed with species specific PNS for BVDV 1, BVDV 2, BDV, CSFV and with the new proposed taxons. The last comparison was performed to obtain genotype determination within a selected species.

A html file was produced at the end of the procedure which is useful for storing and printing the results. This showed the three palindromic fractions of the sequence, V1, V2 and V3, and the related parameters for genus, species and genotype characterization (Figs. 2 and 3).

The secondary structure construction showed results with slight differences and with more precision at the loop level than those obtained by Genetyx-Mac software, based on the algorithm of Zuker and Stiegler (1981) with minimum free energy calculated according Freier et al. (1986). For example, the BVDV-2 strain BS-95-II V1 locus presented a loop consisting of 9 nucleotides (5′-AUCAGUUGA-3′). This sequence was reorganized with a loop reduced to four nucleotides (5′-AGUU-3′), followed by two strong binding base pairs G–C and A–U and an unpaired adenine, resulting in an irregular palindromic shape, when calculated by Genetyx-Mac (not shown). However, this discrepancy was rare and in general the two applications corresponded. In the Giraffe strain, the V1 locus obtained by Genetyx-Mac showed the correct palindromic shape and the two potential strong bindings A–U at the level of the loop were not applied, avoiding a similar alteration as in BS-95-II.

The construction of the V2 palindrome did not present any particular difficulties due to the strictly conserved nucleotide sequences in the locus. The variability of the nucleotide number for the other two palindromic structures V1 and V3 was in contrast, an element that required particular attention. However, the resulting structures were correct and showed a relation to the expected genus characteristics. Table 4 shows an example of the construction of the V3 palindrome locus with the determination of the length of the sequence. Only in four cases, the construction of V3 for the BVDV-1 strains zvr711, 1248/01, G and W was problematic due to specific and uncommon aspects of their sequences.

The V1 was the last identified palindrome. Particular aspects had to be taken into account. The number of nucleotides included in the palindrome was variable, mainly 39 and up to 42–44 in some BVDV 2 and BDV strains, and the Giraffe strain. The starting point of the V1 region in the genomic sequence was variable depending on the type of primers used for the RT-PCR reaction. In order to construct the correct V1 palindrome, the specified condition for a minimum number of nucleotides between the C of the characteristic bulge (5′-nG/A/CnACn.nCUnG/An-3′), and between the V1 and V2, had to be applied. This parameter was necessary to avoid the construction of incoherent V1 palindromes, since some strains