ACTA VET. BRNO 2016, 85: 113-119; doi:10.2754/avb201685020113

The expression and serological reactivity of recombinant *canine herpesvirus 1* glycoprotein D

Markéta Vaňková[1,2], Dobromila Molinková[1,2], Vladimír Celer[1,2]

University of Veterinary and Pharmaceutical Sciences Brno, Faculty of Veterinary Medicine,
[1]Institute of Infectious Diseases and Microbiology, [2]CEITEC – Central European Institute of Technology,
Brno, Czech Republic

Received January 16, 2016
Accepted May 2, 2016

Abstract

The aim of this work was to express recombinant glycoprotein D of *canine herpesvirus 1* in bacterial cells and to evaluate its diagnostic sensitivity and specificity when compared to traditional serological methods. The gene fragment coding glycoprotein D of *canine herpesvirus 1* was amplified by polymerase chain reaction, cloned into plasmid vector and expressed in *Escherichia coli* cells. Recombinant protein was then purified and used as an antigen in immunoblot for a detection of *canine herpesvirus 1* specific antibodies. Antibody testing was performed on the panel of 100 canine sera by immunoblot with recombinant glycoprotein D as antigen and compared with indirect immunofluorescence assay. Serum samples were collected from 83 dogs with no history of *canine herpesvirus 1* or reproductive disorders, and from 17 dogs from breeding kennels with a history of *canine herpesvirus 1* related reproductive disorders. Sensitivity of glycoprotein D based immunoblot was 89.2% and specificity was 93%. Kappa value was calculated to be 0.8 between immunoblot and indirect immunofluorescence assay. Antibodies against *canine herpesvirus 1* infection were detected in 33% of samples by immunoblot assay. Our study confirms that recombinant glycoprotein D expressed in bacterial cells could be used as a suitable and sensitive antigen for immunological tests and that herpesvirus infection seems to be common among the canine population in the Czech Republic.

Viral infection, dog, plasmid, Escherichia coli, recombinant protein, immunoblot

Canine herpesvirus 1 (CHV-1) is a member of the *Herpesviridae* family, with a host range restricted to the *Canidae* family. The virus was described for the first time by Carmichael (1965) as the cause of death in newborn puppies. Transmission occurs through direct contact with body fluids of infected animals, but venereal and transplacental transmission is also possible. *Canine herpesvirus 1* can cause acute and usually fatal systemic haemorrhagic disease in litters of neonatal puppies. Affected puppies generally die within a few days. Infection by CHV-1 during mating and pregnancy can also implicate infertility, embryonic death, mummification, abortion, birth of weak pups and stillbirth. However, in adult dogs, the virus usually causes subclinical infection or only mild clinical signs of the upper respiratory tract, genital tract or eye (Hashimoto et al. 1983; Carmichael and Green 1998).

Direct detection of CHV-1 is possible during the phase of shedding of the virus from mucosal swabs, as well as from organs of latently infected animals or organs of dead puppies by various modifications of the polymerase chain reaction (PCR) technique (Burr et al. 1996; Miyoshi et al. 1999; Decaro et al. 2010). Moreover, additional methods such as *in situ* hybridisation and virus cultivation on tissue cultures can be used (Schulze et al. 1998).

Canine herpesvirus 1 is known to be poorly immunogenic. The titre of neutralizing antibodies increases rapidly for 1–2 weeks following infection or vaccination. High titres of antibodies

Address for correspondence:
MVDr. Markéta Vaňková
Institute of Infectious Diseases and Microbiology
University of Veterinary and Pharmaceutical Sciences Brno
Palackého tř. 1946/1, 612 42 Brno
Czech Republic

Phone: +420 541 562 280
E-mail: vankova.marketa@email.cz
http://actavet.vfu.cz/

are detected only after approximately two months; however, very low titres of antibodies may be present for up to two years. The level of antibodies can also be regularly stimulated by reactivation of the latent virus. However, seropositivity is a confirmation of viral exposure, not acute infection or shedding of the virus (Carmichael and Green 1998).

Epidemiological data based on seroprevalence studies performed in different countries, reported seropositivity of about 13.4–94% (Reading et al. 1998; Rijsewijk et al. 1999; Pratelli et al. 2014). *Canine herpesvirus 1* is a common infection in populations of domestic and wild dogs, especially in dogs living in colonies (Reading and Field 1999). High titres of neutralizing antibodies were confirmed for example in Norway and Finland (Dahlbom et al. 2009; Krogenæs et al. 2012). However, in some countries such as Italy, lower seroprevalence (13.4%) was reported (Pratelli et al. 2014).

Current serological tests (e.g. virus neutralization test, indirect immunofluorescent test) are based on whole virus particles as antigen, however, the use of more sensitive immunoenzymatic tests (ELISA) requires the use of purified recombinant virus proteins. Based on the similarity with other herpesviruses and as shown by experimental data, glycoprotein D (gD) of CHV-1 induces immune response in infected animals and also bears several neutralizing epitopes (Xuan et al. 1991; Maeda et al. 1997) and can thus represent suitable antigen for serological testing.

The main goal of this work was to express recombinant glycoprotein D of CHV-1 in *Escherichia coli* cells and to compare diagnostic sensitivity and specificity of the developed test on a panel of canine sera from dogs with different CHV-1 status.

Materials and Methods

Serum samples

Evaluation of recombinant protein was performed on panels of 100 canine sera originating from clinically healthy animals and from animals with a history of CHV-1 related reproductive disorders. Examined dogs had never been vaccinated against CHV-1.

Clinically healthy dogs

Eighty-three sera originated from healthy dogs without any clinical records of CHV-1 infection.

Animals from breeding kennels

A total of 17 animals from two breeding kennels were sampled for this serological comparison.

One kennel (12 serum samples) kept English and French bulldogs with a recent history of reproductive failures. The reproductive problems described were infertility, stillbirth, birth of weak pups, and death of puppies a few days after birth. Two animals had lost their litters during previous pregnancy. The presence of CHV-1 associated problems in the breeding colony was confirmed by CHV-1 detection by PCR in the parenchymatous organs of one of the dead puppies.

In the other breeding kennel, 5 serum samples were taken from Labrador retrievers with reproductive failures (four females, one male). The virus was detected by PCR in the parenchymatous organs of one of the puppies which had died in the third week after birth.

Amplification and cloning of gD

Virus deoxyribonucleic acid (DNA) was extracted from CHV-1 (F 205 strain, ATCC® VR-1785™) cultivated on Madin-Darby canine kidney (MDCK) epithelial cell line. Virus DNA was purified from 100 µl of cell culture supernatant using NucleoSpin Tissue kit (Macherey Nagel, Germany) according to the instructions of the manufacturer. The DNA obtained was quantified by spectrophotometry and stored at -20 °C until use.

The gene fragment coding the glycoprotein D of CHV-1 was amplified by polymerase chain reaction (PCR) using a pair of primers:

Sense: TATAAATGGGTAGACCCTC

Anti-sense: ATTTGTATTTTTAAAATAATCTTCAAGATT

Primers were designed according CHV-1 sequence available at the GenBank (CHU84223).

Stop codon was contained within the antisense primer. The PCR conditions included a 4 min denaturation step at 94 °C followed by 30 cycles of 45 s at 94 °C, 45 s at 50 °C, and 45 s at 72 °C. A final extension for 30 min at 72 °C was then performed to add a single deoxyadenosine (A) to the 3′ ends of PCR products.

The PCR product was inserted into the plasmid vector TrcHis (Invitrogen) by T/A cloning strategy and the resulting construction (TrcHis/gD) was used to transform competent *E. coli* cells (strain TOP 10). Transformed cells were then inoculated on Luria-Bertani agar (LB agar) supplemented with 1% glucose and ampicillin

(100 mg/ml) and incubated at 37 °C for 18 h. Isolation of the plasmid DNA (TrcHis/gD) was performed by QIAGEN Plasmid Mini Kit (QIAGEN GmbH, Germany) according to the instructions of the manufacturer. Subsequently, the TrcHis/gD construct was verified by sequencing.

Expression of recombinant protein (gD)

Expression of recombinant protein was performed in *Escherichia coli* cells (strain TOP10). Bacterial cells were grown in 20 ml of Luria-Bertani broth (LB broth) supplemented with 1% glucose and ampicillin (100 μg/ml) with constant shaking (37 °C for 16 h). Cells were then centrifuged and the pellet resuspended in 500 ml of LB broth with ampicillin (100 μg/ml) and grown until the optical density of a sample measured at a wavelength of 600 nm (OD_{600}) reached 0.5 at 37 °C at a shaking speed of 250 rpm. The expression of recombinant protein was induced by 100 mM isopropyl-β-D thiogalactopyranoside (IPTG) for 4 h at 37° C. Bacterial suspensions were centrifuged at 3,400 *g* for 20 min and the pellet stored at -20 °C until use.

Purification of recombinant protein (gD)

Recombinant protein was purified by immobilized-metal affinity chromatography (IMAC) using polyhistidine tag under denaturing conditions from the insoluble fraction of bacterial lysate.

After induction, the bacterial pellet was resuspended in phosphate buffered saline (PBS) with lysozyme (200 mg/ml) and incubated for one hour on ice. Subsequently, the cell lysate was homogenized by sonication (4 pulses for 20 s, intervals between pulses of 2 min with continuous cooling on ice). The homogenized suspension was centrifuged at 4 °C at 5,000 *g* for 20 min. The pelleted material was resuspended in 40 ml of denaturing Tris buffer (20 mM Tris pH 7.8, 1 M sodium chloride, 0.1% Tween 20, 8 M Urea and 10 mM imidazole) and incubated at room temperature for 1 h. Then 1 ml of equilibrated Ni-NTA Agarose (Qiagen) was added to dissolved material and incubated for 1 h at room temperature on a horizontal shaker. The agarose was then washed with 200 ml of denaturing buffer containing 20 mM imidazole; recombinant protein was then eluted by denaturing buffer supplemented with 300 mM imidazole. Individual 1 ml fractions were collected. Presence and purity of recombinant protein in the fractions was analysed by sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) and by immunoblot. The concentration of recombinant protein was spectrophotometrically determined and the protein was stored at -80 °C until use.

SDS-PAGE and immunoblot

The expression and purification of recombinant protein was analysed by SDS-PAGE. Identity of recombinant protein was then confirmed by incubation with monoclonal anti-polyhistidine antibody and then with known positive control serum.

The protein eluate was mixed with Laemmli sample buffer and heated to 95 °C in a water bath for 4 min. Subsequently, it was applied to wells in polyacrylamide gel (12% resolving and 4% stacking polyacrylamide gel), separated by electrophoresis in Tris-glycine buffer and stained with Coomassie Brilliant Blue.

Purified protein (gD) was blotted onto nitrocellulose membrane after its separation by SDS-PAGE. Nitrocellulose strips were blocked with 3% nonfat dry milk in PBS pH 7.3 (MPBS) for 1 h and then washed in PBS.

Sera were diluted 1:100 in MPBS-T (PBS with 1% Tween 20, 0.5 M sodium chloride and 3% non-fat dry milk) and incubated with nitrocellulose strips for 1 h. Bound antibodies were detected with anti-dog IgG alkaline phosphatase conjugate (Sigma - Aldrich, Czech Republic) diluted 1:2000 in MPBS-T. The reaction was then made visible with 5-bromo-4-chloro-3-indolyl-phosphate, p-toluidine salt / nitro blue tetrazolium chloride (NBT/BCIP; Sigma - Aldrich, Czech Republic). Between incubation steps, strips were washed (3 × 5 min) in MPBS-T.

Indirect immunofluorescence assay (iIFA)

For confirmation of serological status, indirect immunofluorescence assay was used (VMRD, Inc., USA). Sera for iIFA test were diluted 1:100 in dilution buffer and tested according to the instructions of the manufacturer.

Statistic

Kappa value to evaluate agreement between immunoblot and iIFA was determined by Landis and Koch (1977).

Specificity and sensitivity of immunoblot was calculated as follows:
Sensitivity = TP/(TP + FN) × 100
Specificity = TN/(TN + FP) × 100
Where: TP, FN, TN and FP stand for true-positive, false-negative, true-negative and false-positive, respectively (Barrouin-Melo et al. 2007).

Results

The 828 bp long DNA fragment of CHV-1 glycoprotein D was PCR amplified and cloned into expression vector pTrcHis. Recombinant protein (gD) was expressed from a single bacterial colony following induction by isopropyl-β-D-thiogalactopyranoside

(IPTG). Protein with appropriate molecular weight of 34 kilodalton (including N′ terminal polyhistidine) was detected on SDS-PAGE gel. As the recombinant protein was present mainly in the insoluble fraction of bacterial lysate (Fig. 1), purification from inclusion bodies was performed by immobilized-metal affinity chromatography (IMAC) using polyhistidine tag under denaturing conditions. Level of protein purification and presence of polyhistidine tag was assessed by SDS-PAGE and immunoblot.

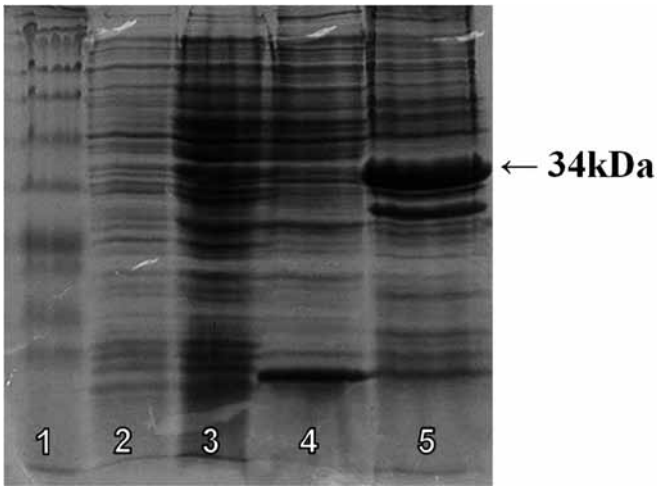

Fig. 1. Sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) - expression of recombinant gD.

Lane 1 - molecular weight marker; lane 2 – cell lysate before induction; lane 3 – cell lysate following induction; lane 4 - soluble fraction of cell lysate following induction; lane 5 - insoluble fraction of cell lysate following induction; kilodalton (kDa)

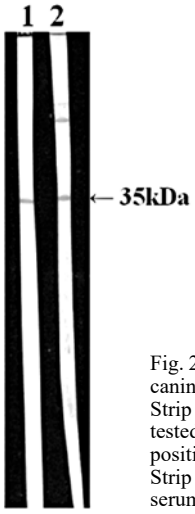

Fig. 2. Immunoblot with canine sera
Strip 1 - one of the tested canine sera - positive reaction
Strip 2 - control positive serum; kilodalton (kDa)

Purified recombinant protein was used as the antigen for detection of CHV-1 specific antibodies by immunoblot. Serological reactivity of the protein was confirmed by CHV-1 positive control serum (Fig. 2).

The panel of 100 canine sera samples was tested for the presence of CHV-1 specific antibodies by immunoblot and compared with indirect immunofluorescence assay (Table 1). Although both tests gave identical results on 91% of examined sera, 9% of sera were discrepant. Kappa value for this comparison was calculated to be 0.8 which approximates almost perfect agreement between immunoblot and iIFA. Sensitivity and specificity of gD based immunoblot was 89.2% and 93% respectively.

The prevalence of CHV-1 infection in dogs with no previous history of infection and in kennels with diagnosed CHV-1 related reproductive disorders is shown in Table 2.

Table 1. Comparison of immunoblot and indirect immunofluorescence assay.

		Indirect immunofluorescence assay		
		Positive	Negative	Total
Immunoblot (glycoprotein D of CHV-1)	Positive	29 (29%)	4 (4%)	33 (33%)
	Negative	5 (5%)	62 (62%)	67 (67%)
	Total	34 (34%)	66 (66%)	100 (100%)

Table 2. Comparison of prevalence of *canine herpesvirus* 1 (CHV-1) with different infection statuses.

Status of infection	Positive	Negative
Healthy dogs	25 (30.2%)	58 (69.8%)
Kennels with a confirmed CHV-1	8 (47.06%)	9 (52.94%)

Serological prevalence in breeds with reproductive disorders was significantly higher (47%) compared to the prevalence in the healthy dog population (30%) (Table 2).

Discussion

Canine herpesvirus infection is one of the important topics of current veterinary medicine, as CHV-1 is an important reproductive pathogen in dogs. The diagnostics of the disease is performed mainly by direct detection of the virus during clinical manifestation. Detection of antibodies is used in epidemiological studies to monitor the prevalence of the disease. Advanced, more sensitive serological tests based on recombinant virus glycoproteins as antigens are required for better understanding of CHV-1 disease pathogenesis.

The usefulness of gD as the antigen for serological tests results from its biological properties. It is considered a structure indispensable for the virus replication cycle during attachment and subsequent penetration into a permissive cell, as could be deduced from analogy in other alphaherpesviruses (Ligas et al. 1988; Rauh et al. 1991). Glycoprotein D is also responsible for induction of neutralizing antibodies due to the presence of neutralizing epitopes (Maeda et al. 1997). Also in other herpesvirus infections, gD was successfully used as antigen in diagnostic tests (Meskhat et al. 2012; Fuentealba et al. 2014).

We have successfully expressed gD of CHV-1 in bacterial *E. coli* expression system. Although the resulting protein was not glycosylated due to the inability of *E. coli* to perform post translation modifications characteristic for eukaryotic cells, it was recognized by CHV-1 specific antibodies in immunoblot. This finding suggests that structures recognized by at least some of these antibodies are most likely linear, non-glycosylated epitopes. Li et al. (2002) and Mosko et al. (2004) successfully used non-glycosylated protein for diagnostic purposes which confirm that virus proteins produced in the *E. coli* system can supply a cheap and valuable source of diagnostic antigens. Currently, one of the most widely used tests for serological diagnostic of CHV-1 specific antibodies is indirect immunofluorescent test (Pratelli et al. 2014). Comparison of iIFA and immunoblot based on recombinant gD gave comparable results on a panel of 100 canine sera with only nine sera giving discordant results.

Five sera were positive by iIFA, but gave a negative result in immunoblot. In this case we can speculate that the superior sensitivity of iIFA is due to the presence of additional virus structures reacting with antibodies in the iIFA. Four of the tested sera were found to be

positive by immunoblot and negative by iIFA, which can be explained by generally higher sensitivity of immunoenzymatic tests.

The sensitivity and specificity of the gD-based test was determined to be 89.2% and 93%, respectively, compared to iIFA. Despite these discrepant results, the calculation of kappa value approached almost perfect agreement between the two tests. In the following part of this study, recombinant gD was used for serological screening of CHV-1 antibodies (Landis and Koch 1977).

The prevalence of CHV-1 specific antibodies in the Czech Republic was never determined in previous years. The results of the present study, based on the testing of a limited set of canine sera, revealed the prevalence of infection to be 33–35% depending on the test used. This value is in close agreement with results obtained by other authors estimating a CHV-1 infection prevalence of 13.4–94% in different European countries (Reading et al. 1998; Rijsewijk et al. 1999; Pratelli et al. 2014).

However, the prevalence of CHV-1 infection determined in our study was likely influenced by biased sampling; a significant proportion of samples (17%) originated from breeding kennels where reproduction failure was diagnosed in the recent past and seropositivity in these kennels reached 47%. Confirmation of higher seropositivity in these kennels requires further studies performed on higher numbers of animals kept under different conditions and with different health status (Ronsee et al. 2004; Dahlbom et al. 2009).

The conclusion can be drawn from our study that recombinant gD expressed in *E. coli* cells could be used as a suitable and sensitive antigen for immunological tests and that the prevalence of the infection in the Czech Republic is comparable to the prevalence determined in other countries.

Acknowledgements

This work was supported by project CZ.1.07/2.3.00/30.0014, and by "CEITEC – Central European Institute of Technology" (CZ.1.05/1.1.00/02.0068) from the European Regional Development Fund and project 37/2011/FVL IGA VFU Brno.

Referents

Barrouin-Melo SM, Poester FP, Ribeiro MB, de Alcântara AC, Aguiar PH, Nascimento IL, Schaer RE, Nascimento RM, Freire SM 2007: Diagnosis of canine brucellosis by ELISA using an antigen obtained from wild *Brucella canis*. Res Vet Sci **83**: 340-346

Burr PD, Campbell MEM, Nicolson L, Onions DE 1996: Detection of canine herpesvirus 1 in a wide range of tissues using the polymerase chain reaction. Vet Microbiol **53**: 227-237

Carmichael LE, Squire RA, Krook L 1965: Clinical and pathologic features of a fatal viral disease of newborn pups. Am J Vet Res **26**: 803-814

Dahlbom M, Johnsson M, Myllys V, Taponen J, Andersson M 2009: Seroprevalence of canine herpesvirus-1 and *Brucella canis* in Finnish breeding kennels with and without reproductive problems. Reprod Domest Anim **44**: 128-131

Decaro N, Amorisco F, Desario C, Lorusso E, Camero M, Bellacicco AL, Sciarretta R, Lucente MS, Martella V, Buonavoglia C 2010: Development and validation of a real-time PCR assay for specific and sensitive detection of canid herpesvirus 1. J Virol Methods **169**: 176-180

Fuentealba N, Sguazza G, Scrochi M, Bravi M, Zanuzzi C, Corva S, Gimeno E, Pecoraro M, Galosi C 2014: Production of equine herpesvirus 1 recombinant glycoprotein D and development of an agar gel immunodiffusion test for serological diagnosis. J Virol Methods **202**: 15-18

Greene CE, Carmichael LE 2006: Canine herpesvirus infection. In: Greene CE (3rd Ed.): Infectious Diseases of the Dog and Cat. MO: Elsevier, St. Louis, pp. 47-53

Hashimoto A, Hirai K, Suzuki Y, Fujimoto Y 1983: Experimental transplacental transmission of canine herpesvirus in pregnant bitches during the second trimester of gestation. Am J Vet Res **44**: 610-614

Krogenæs A, Rootwelt V, Larsen S, Sjøberg EK, Akselsen B, Skår TM, Myhre SS, Renström LH, Klingeborn B, Lund A 2012: A serological study of canine herpes virus-1 infection in the Norwegian adult dog population. Theriogenology **78**: 153-158

Landis JR, Koch GG 1977: The measurement of observer agreement for categorical data. Biometrics **33**: 159-174

Li M, Li X, Liu M 2002: Cloning and expression of HSV-I, II type-common antigen gD in *Escherichia coli*. Zhonghua Shi Yan He Lin Chuang Bing Du Xue Za Zhi **16**: 176-178

Ligas MW, Johnson DC 1988: A herpes simplex virus mutant in which glycoprotein D sequences are replaced by beta-galactosidase sequences binds to but is unable to penetrate into cells. J Virol **62**: 1486-1494

Maeda K, Yokoyama N, Fujita K, Xuan X, Mikami T 1997: Role of one N-linked oligosaccharide chain on canine herpesvirus gD in its biological activity. J Vet Med Sci **59**: 1123-1128

Meshkat Z, Roostaee MH, Soleimanjahi M, Zandi K 2012: Evaluation of antibodies against glycoprotein D (gD) and glycoprotein G (gG) in HSV-1 infected individuals' serum samples. Eur Rev Med Pharmacol Sci **16**: 499-502

Miyoshi M, Ishii Y, Takiguchi M, Takada A, Yasuda J, Hashimoto A, Okazaki K, Kida H 1999: Detection of canine herpesvirus DNA in the ganglionic neurons and the lymph node lymphocytes of latently infected dogs. J Vet Med Sci **61**: 375-379

Mosko T, Kosovský J, Rezuchová I, Durmanová V, Kúdelová M, Rajcáni J 2004: Expression of herpes simplex virus 1 glycoprotein D in prokaryotic and eukaryotic cells. Acta Virol **48**: 97-107

Pratelli A, Colao V, Losurdo M 2014: Serological and virological detection of canine herpesvirus-1 in adult dogs with and without reproductive disorders. Vet J **200**: 257-260

Rauh I, Mettenleiter TC 1991: Pseudorabies virus glycoproteins gII and gp50 are essential for virus penetration. J Virol **65**: 5348-5356

Reading MJ, Field HJ 1998: A serological study of canine herpes virus-1 infection in the English dog population. Arch Virol **143**: 1477-1488

Reading MJ, Field HJ 1999: Detection of high levels of canine herpes virus-1 neutralising antibody in kennel dogs using a novel serum neutralisation test. Res Vet Sci **66**: 273-275

Rijsewijk FAM, Luiten EJ, Daus FJ, Van der Heijden RW, Van Oirschot JT 1999: Prevalence of antibodies against canine herpesvirus 1 in dogs in The Netherlands in 1997–1998. Vet Microbiol **65**: 1-7

Ronsse V, Verstegen J, Onclin K, Farnir F, Poulet H 2004: Risk factors and reproductive disorders associated with canine herpesvirus-1 (CHV-1). Theriogenology **61**: 619-636

Schulze C, Baumgärtner W 1998: Nested polymerase chain reaction and in situ hybridization for diagnosis of canine herpesvirus infection in puppies. Vet Pathol **35**: 209-217

Xuan X, Horimoto T, Limcumpao JA, Takumi A, Tohya Y, Takahashi E, Mikami T 1991: Neutralizing determinants of canine herpesvirus as defined by monoclonal antibodies. Arch Virol **116**: 185-195