TÜBİTAK

Turkish Journal of Veterinary and Animal Sciences

http://journals.tubitak.gov.tr/veterinary/

Research Article

Turk J Vet Anim Sci
(2023) 47: 215-221

doi:10.55730/1300-0128.4288

Examination of canine herpesvirus-1 in dogs by serological, molecular, and pathological methods

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Received: 01.03.2023 • **Accepted/Published Online:** 01.01.2023 • **Final Version:** 13.06.2023

Abstract: In this study, it was aimed to examine Canine Herpesvirus-1 (CHV-1) with different diagnostic methods and investigate the pathological changes in positive samples. For this purpose, 100 dog uteruses of different ages and breeds, were removed by ovariohysterectomy and their blood samples were used as the study material. Enzyme-linked immunosorbent assay (ELISA) and Indirect Fluorescent Antibody Test (IFAT) for serological diagnosis, polymerase chain reaction (PCR) method for molecular diagnosis, immunohistochemistry (IHC) and immunofluorescence (IF) staining for immunopathological diagnosis were performed on the samples. Additionally, hematoxylin and eosin (HE) staining was performed to examine the histopathological changes in the CHV-1-positive samples. An antibody presence rate of 37% was detected by both ELISA and IFAT tests. The immunohistochemical and immunofluorescent staining results revealed the presence of antigens at rates of 33% and 35%, respectively. No positivity was recorded in any of the samples by the PCR method. In the histopathological examinations of the CHV-1-positive uterus samples, it was observed that 12 had catarrhal endometritis, 10 had purulent endometritis, 8 had chronic nonpurulent endometritis and 7 were healthy. No lesions were found in the other 63 samples that were not CHV-1 positive. In conclusion, it was determined that the best methods for the diagnosis of CHV-1 infection were ELISA and IFAT, followed by immunofluorescence and immunohistochemical staining. The positive samples mostly showed catarrhal endometritis, and the presence of the virus was also detected in healthy tissues. In this study, the prevalence of CHV-1 in dogs was determined for the first time with a variety of diagnostic methods, and histopathological changes in naturally infected specimens were investigated.

Key words: Canine Herpesvirus-1, ELISA, IFAT, immunohistochemistry, immunofluorescence, PCR

1. Introduction

Canine Herpesvirus-1(CHV-1) infection is one of the most common diseases in domestic and wild dogs worldwide. The disease, which usually has a systemic and fatal course in animals younger than three weeks of age, causes respiratory, urinary, genital, ocular, and neurological disorders at higher rates in adults [1,2]. Nonhygienic environments, malnutrition, other diseases, and various stress factors leading to immunosuppression play a role in the etiology of the disease. CHV-1 is a DNA virus belonging to the *Alphaherpesvirinae* subfamily under the *Herpesviridae* family. Considering that the temperatures convenient for virus growth are 35–36 °C, hypothermia is an important factor in the formation of infection. Puppies that are more susceptible to the disease have a worse prognosis and a higher mortality rate. In adults, the disease is usually asymptomatic, and the mortality rate is low. Infection is transmitted by contact (saliva, vaginal secretion) and transplacental routes [3–5]. The agent that enters the body first multiplies in the mucosal epithelium, and there, it infects macrophages as well, leading to primary viremia by entering the systemic circulation. Afterwards, the agent attaches to lymphoid tissues, especially to the uterus, spleen, liver, kidneys, and lungs. It causes widespread multifocal necrosis, necrotizing vasculitis, and thrombocytopenia in these regions, resulting in hemorrhagic syndrome. In adults, although the infection is subclinical, lesions can be observed in the upper respiratory tract, genital and urinary systems, as well as the eyes [2,6]. Like other herpes viruses, in CHV-1 infection, the agent can reside latently in the trigeminal and lumbosacral ganglia, tonsils, and parotid salivary glands after primary infection. In cases of immunosuppression, the latent agent may cause reinfection [7,8]. Various diagnostic methods are used to diagnose the

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disease, showing the presence of antigens or antibodies in the blood, fresh tissue, and swab samples. For the diagnosis of the disease, serum neutralization (SN), ELISA, IFAT, Direct Fluorescent Antibody Test (DFAT), virus isolation, electron microscopy, PCR, and immunohistochemical methods are used [5,9,10].

CHV-1 is a disease with serious consequences on the uterus health of canines and its prevalence has been increasing lately. To manage the disease, it is necessary to determine the most appropriate and practical diagnostic method in the determination of its prevalence. For this reason, in this study, the prevalence of CHV-1 in dogs was demonstrated by different diagnostic methods, trying to determine the most ideal one. Additionally, histopathological examinations of positive samples were performed to contribute to the knowledge of the pathogenesis of the disease.

2. Material and method

2.1. Animal material

This study was conducted with the approval of Atatürk University Animal Experiments Local Ethics Committee, dated 12.06.2018, and numbered 2018/65. The study materials consisted of 100 uteruses of unvaccinated dogs of different ages and breeds removed by ovariohysterectomy at Atatürk University, Faculty of Veterinary Medicine, Animal Hospital, as well as their blood samples. While some of the removed uterus tissues were kept in 10% Neutral Buffer Formalin (NBF) for histopathological (HE) and immunopathological (IHC, IF) examinations, some were stored at –80 °C for molecular (PCR) examinations. Before the operation, blood samples taken through the cephalic and jugular veins were centrifuged at 1200 g for 10 min, and their sera were extracted. The sera to be used in serological (ELISA, IFAT) studies were placed in Eppendorf tubes, and stored at –80 °C. Since these methods are the most widely used diagnostic methods recently, it was preferred to evaluate them.

2.2. Histopathology

Dehydration, transparentizing, and paraffinization processes were consecutively applied to the uterus tissues which were fixated in a formalin solution, and routine tissue follow-ups were performed. Then, 5 µ thick sections from each of the samples which were embedded in paraffin blocks were taken onto normal and adhesive slides with a rotary microtome (Leica RM 2235, Nussloch, German). After HE staining the sections were examined under a light microscope (Leica DM 2500, Nussloch, German). According to their findings, the samples were evaluated as catarrhal endometritis, purulent endometritis, and chronic nonpurulent endometritis, and those with no lesions were considered healthy.

2.3. Immunohistochemical staining

Deparaffinization and dehydration processes were applied to the tissues taken onto adhesive slides. For endogenous peroxidase inactivation, the tissues were treated with 3% H2O2. Tissues were boiled in a microwave oven with antigen retrieval solution to reveal any presence of antigens. A protein block solution was dripped onto the tissues to prevent nonspecific antigen binding. The tissues were incubated at 37 °C with the primary antibody (Cat No: SLD-IFA-CHV; VMRD, Pullman, USA) reconstituted at a ratio of 1:50. Then, the tissues were incubated first with biotinylated secondary antibody and then with the streptavidin-peroxidase conjugate. To demonstrate the reactions on the tissues, 3-3' Diaminobenzidine (DAB) chromogen was dripped onto sections, and after counterstaining with Mayer's hematoxylin, the preparations were examined under a light microscope (Leica DM 2500, Nussloch, German).

2.4. Immunofluorescent staining

After applying the same procedures including primary antibody incubation, as in immunohistochemical staining, immunofluorescent antibody (Anti-Canine IgG (FITC), Code: CJ-F-CANG-AP, VMRD, Pullman, USA) reconstituted at 1:20 was dripped onto the tissue sections and incubated for 45 min in a dark environment. Then, the preparations, which were covered with a coverslip by dripping glycerol/distilled water (1:9) solution on the tissues, were examined under a fluorescence microscope (Zeıss Axıo Scope A1, Göttingen, German).

2.5. PCR

Uterine tissues homogenized in PBS for viral nucleic acid isolation. For PCR first, DNA extraction was performed with the PureLink Genomic DNA mini kit (Catalog no: 2024278, Invitrogen, USA) using the manufacturer's protocol on the homogenized uterus tissues. To examine CHV-1 DNA in samples with isolated nucleic acid, the primer pairs used by Monteiro et al. [11] were adopted as references. Primer sequences are listed in Table 1. Both positive (Canine herpesvirus 1 VR-552) and negative (double distilled water) controls were used in the PCR application. DNA dye was added to the PCR

Table 1. Primer sequences, gene region, and product sizes.

Region	Primers	sequences F/R(5'-3')	bp	Reference
CHV-1 gb	CHV-1gB CHV-1gB	**F:** CCTAAACCTACTTCGGATGA **R:** GGCTTTAAATGAACTTCTCTGG	450	11

products obtained by this reaction, the products were electrophoresed in 0.7% agarose gel and were visualized under a UV transilluminator.

2.6. ELISA

In the ELISA test, after the serum samples frozen in Eppendorf tubes were adjusted to room temperature, a commercial ELISA kit (EVL™ Woerden, Holland) was used to detect the presence of CHV-1 antibodies (IgG). Finally, the optical densities of the samples on the plate were read with the help of a spectrophotometer (Thermo Scientific, Multiskan GO, USA) in a microplate with a 450 nm filter.

2.7. IFAT

For the IFA test, the frozen serum samples were adjusted to room temperature and diluted with buffered saline at a ratio of 1:80. Then, the IFA test kit (FluoHerpesvırus Canine, Cat No. 27262610, Agrolabo, Italy) was used on the samples in accordance with the manufacturer's recommendations to determine CHV-1 antibodies (IgG) presence. Two to three drops of glycerin buffer were dropped onto the slides on which antigens resided, and the kit protocol was applied. The samples were then covered with a coverslip. Finally, the preparations were examined under the ×400 lens of the fluorescence microscope (Zeıss Axıo Scope A1, Göttingen, German).

3. Results

3.1. Histopathological findings

According to the histopathological findings observed on the uterus tissues, the samples were classified as catarrhal, purulent, and chronic nonpurulent endometritis. No pathologic findings were found in some positive samples. The samples that did not have any lesions in their uterus sections were considered healthy. In the samples with catarrhal endometritis, hyperemia, edema, neutrophil leukocytes, and a small number of infiltrating mononuclear cells were detected in the lamina propria of the uterus. Desquamated cells and seromucous exudate-containing leukocytes were observed in the lumen of the uterus (Figure A). In purulent endometritis, numerous neutrophil leukocyte infiltrations were seen in the lamina propria of the uterus, and purulent exudate consisting of desquamated cells, neutrophil leukocytes, and partially mononuclear cells was observed in the lumen (Figure B). In chronic nonpurulent endometritis, lymphoplasmacytic cell infiltration in the lamina propria of the uterus, polypoid hyperplasia in the uterus glands, and localized increases in connective tissue were observed (Figure C). Additionally, basophilic inclusion bodies were found in the epithelium in some of the cases with chronic endometritis (Figure D). According to the diagnostic methods that were applied, the histopathological findings in the CHV-1-positive samples are presented in Table 2.

3.2. Immunohistochemistry findings

In the immunohistochemical staining processes, 33 (33%) of the uterus samples turned out positive. It was observed that the highest degrees of immunopositivity were in macrophages, the mucosal epithelium, and the glandular epithelium, respectively (Figure E, F).

3.3. Immunofluorescence findings

In the immunofluorescent staining processes, 35 (35%) of the uterus samples turned out positive. As in immunohistochemical staining, immunopositivity was found mostly in macrophages, the mucosal epithelium, and the glandular epithelium (Figure G.H).

3.4. PCR findings

Although the herpesvirus template used as a positive control was amplified in the analysis of the uterus tissue homogenates by the PCR method, no positivity was observed in any of the materials used.

3.5. ELISA findings

The presence of CHV-1 antibodies was detected in 37% of the serum samples analyzed using the ELISA test methods.

3.6. IFAT findings

The presence of CHV-1 antibodies was observed in 37% of the serum samples with the IFA test (Figure I).

4. Discussion

Canine herpesvirus infection is one of the major health problems of wild and domestic dogs worldwide. In particular, this infection progresses along with hemorrhagic syndrome in puppies, which is systemic and fatal. In adults, it causes respiratory, urinary, genital, ocular, and neurological disorders. The mortality rate of this infection in adults is not as high as in that puppies. However, in the infection of adults, as in other viral infections, sensitivity to secondary infections increases, and infertility becomes inevitable especially in cases in which the genital organs are affected [2,5,12]. In this study, the prevalence of CHV-1 in adult dogs was determined by various diagnostic methods, and pathological changes in the genital organs were investigated.

It has been reported in the serological studies of CHV-1 on dogs that the prevalence of this infection can vary between 0% and 100% [13–15]. It was stated that the seroprevalence of CHV-1 in kennels in South Africa was 21.5% according to the SN test and 21.6% according to the ELISA test, and there was no significant difference in terms of reliability between the two methods [16]. It was suggested that the seroprevalence of CHV-1 in Mexico was 87%, and the ELISA test provided more reliable results than the SN test [17]. In Romania, CHV-1 antibodies were detected in 86.36% of dog blood sera by the IFA test [18]. In a study conducted using the IFA test in Iran, the seroprevalence of CHV-1 was reported to be 20.7% [19]. In their study in Turkey, Yapici et al. [20] reported that

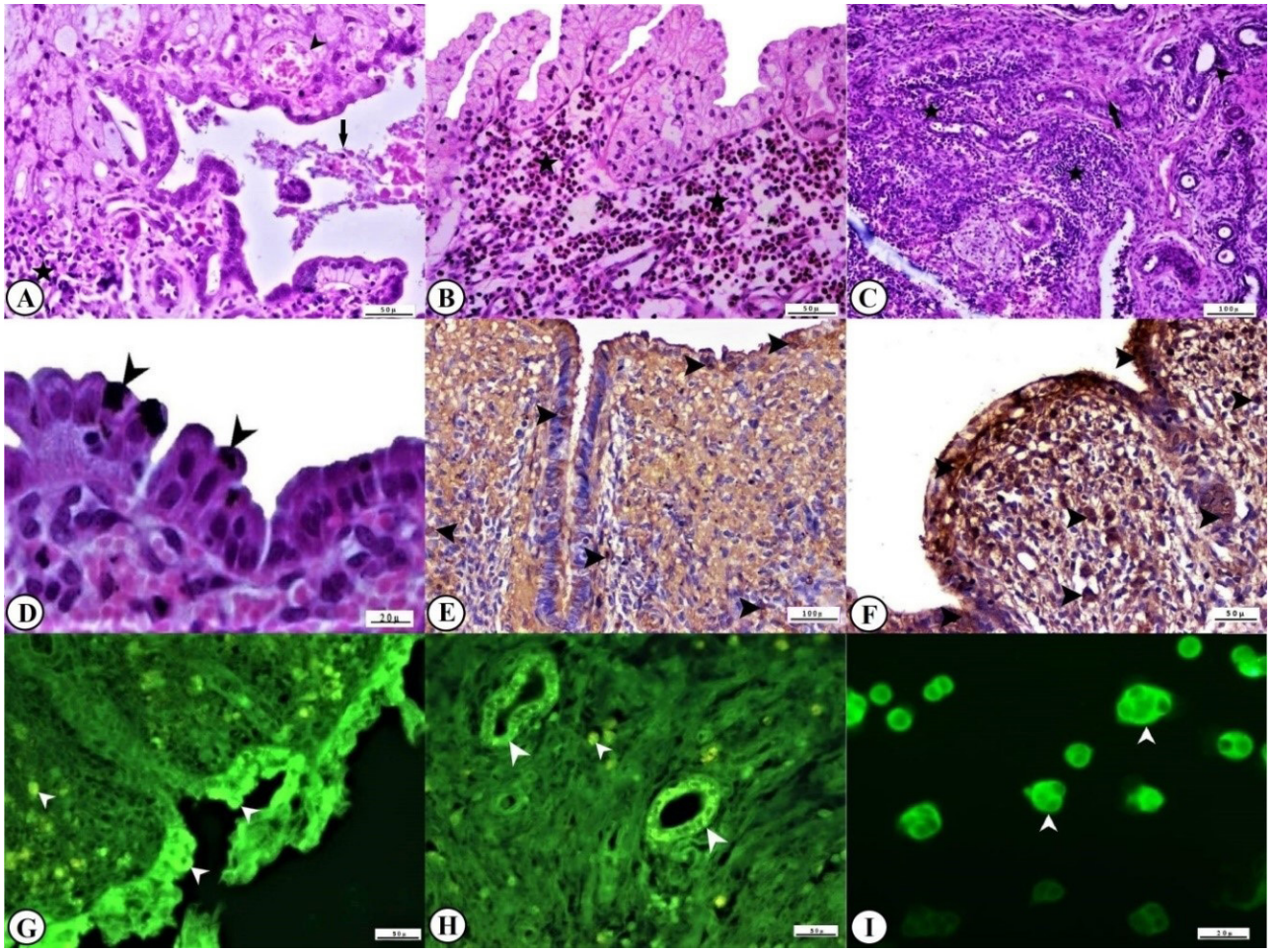

Figure A; Catarrhal endometritis, catarrhal exudate (arrow), inflammatory cell infiltration (star), hyperemia (arrowhead), HE, ×200. B; Purulent endometritis, neutrophil leukocyte cell infiltration (asterisks), HE, ×200. C; Chronic nonpurulent endometritis, mononuclear cell infiltration (stars), polypoid hyperplasia in endometrial glands (arrowhead), fibrous connective tissue formation (arrow), HE, ×100. D; Basophilic inclusion bodies in uterine epithelium, HE, ×400. E; CHV-1 immunopositivity in uterine epithelium and macrophages, IHC, ×100. F; CHV-1 immunopositivity in uterine epithelium and endometrial glands, IHC, ×200. G; CHV-1 immunopositivity in uterine epithelium and inflammatory cells, IF, ×200. H; Immunopositivity in uterine inflammatory cells and endometrial glands, IF, ×200. I; Immunopositivity in CHV-1 loaded epithelial cell lines, IFAT, ×400.

Table 2. Histopathological findings of CHV-1 positive samples according to the diagnostic methods used.

Histopathological findings	IHC	IF	IFAT	ELISA	PCR
Catarrhal endometritis	11	12	12	12	-
Purulent endometritis	9	9	10	10	-
Chronic non-purulent endometritis	7	8	8	8	-
Healthy uterus	6	6	7	7	-
Total	33	35	37	37	-

although they detected 68.79% CHV-1 positivity by the ELISA test in dog serum samples, these samples did not cause morphological changes in MDCK cell cultures and there was no positivity with the DFAT test. In another study in Turkey, a CHV-1 seroprevalence of 39.3% was reported with the ELISA method, and 29.4% seropositivity was reported with a virus neutralization test (VNT) [21]. The same prevalence value was obtained with ELISA and IFAT in the serological tests that were used in this study, in which the seroprevalence of CHV-1 was found to be 37%. In this study, it was determined that the prevalence of CHV-1 in Turkey was common, similar to the findings of Yeşilbağ et al [21].

Apart from serological methods, cell culture, PCR and EM techniques can also be used in the diagnosis of CHV-1 infection. Rezaei et al. [22] detected CHV-1 DNA in 20% of uterus biopsy samples and 10% of vaginal swabs. Pratelli et al. [23] stated that they detected CHV-1 seropositivity at a rate of 14.6% with the SN test and 18.6% with the IFAT test in dog sera, but they found the PCR result negative in vaginal swab samples. Although they amplified the virus DNA sample using PCR, which was the sample they used as a positive control, Bottinelli et al. [24] reported negative PCR for CHV-1 in vaginal swab samples from dogs. In this study, also although the control was positively amplified by the PCR method, no positivity was observed in the tissue analyses similar way. This situation can be explained by the persistence of herpesviruses in the sacral ganglia. As a result of persistent infection, it sends its nucleic acid to the ganglia via neurons. However, the capsid protein remains at the site of infection. Although nucleic acid could not be determined in PCR analysis, the determination of capsid protein with ELISA is due to the virus's life cycle and pathogenesis [25,26].

In the literature review, no studies in which the presence of antigens in uterus tissue sections was demonstrated by immunopathological (IHC, IF) staining methods were encountered. However, Krogenæs et al. [27] detected CHV-1 antibodies in 85.5% of sera which were immune-cytologically collected using the IPMA method. In this study, CHV-1 immunopositivity was detected in 33% of the uterus tissue sections by immunohistochemical staining and 35% by immunofluorescent staining. In both staining methods, antigen positivity was demonstrated mostly in macrophages, the uterus epithelium, and the gland epithelium, respectively. As in the study by Çomaklı et al. [28] the presence of antigens was demonstrated in more samples using the IF method compared to IHC staining. It was observed that there was no significant diagnostic difference between the two methods, but the positivity was more selective with the IF method. It was stated in another study that the IF method had disadvantages such as not providing enough morphological detail as IHC staining did and limited working time due to loss of fluorescence [29]. It was thought that the prevalence value being lower in the immunopathological staining methods of the uterus tissues than that in the serological findings was due to the investigation of the presence of antigens rather than antibodies. These findings supported the knowledge in previous studies that the presence of antibodies persists in the organism for a while after infection [3,30].

In this study, besides prevalence determination by different diagnostic methods, histopathological changes in positive samples naturally infected by CHV-1 were also investigated. Accordingly, it was determined that the CHV-1-positive samples were mostly catarrhal endometritis, purulent endometritis, and chronic nonpurulent endometritis case, respectively. Additionally, it was observed that healthy uterus tissues that did not demonstrate any pathological findings could also be infected. This may be due to the latent nature of the virus or the fact that the agent has not yet created an infection that will create an observable pathological lesion.

5. Conclusions

As a result, it was determined that the best methods for the diagnosis of CHV-1 infection were ELISA and IFAT, followed by IF and IHC staining, respectively. In this study, the prevalence of infection was found to be at a substantial level. A remarkable finding was that the PCR results turned out negative in this study. The presence of some studies with similar findings and the fact that the tissue analyses were negative in this study despite the amplification from positive controls caused the PCR method's effectiveness in the diagnosis of CHV-1 infection to be questioned. Additionally, the histopathological examinations of the specimens naturally infected with CHV-1 revealed that the agent most commonly caused catarrhal endometritis, followed by purulent and chronic nonpurulent endometritis, and the agent could also reside in healthy tissues.

Conflict of interest statement

None of the authors of this article has any conflict of interest.

Acknowledgments

The project was supported by Atatürk University Scientific Research Council (project number: 2018/6536). This study was derived from a thesis (ID: 586052), which was conducted by Fatma Gülten Bayraktar.

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