The first molecular detection of Canine herpesvirus-1 in reproductive specimens of adult dogs in Iran

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Abstract

Background Canine herpesvirus-1 (CHV-1) is recognized to be enzootic in the dog population with a widespread distribution. This pathogen leads to a lethal generalized illness in newborn puppies and is associated with reproductive disorders. CHV-1 should be considered as an important pathogen of neonatal death and infertility; so, it appears to pose a threat for breeding kennels. Although serologic data point to the circulation of CHV-1 among dogs of Iran, not definitive diagnosis has been conducted based on the molecular assay. So, this research was done to detect the prevalence of CHV-1 in dogs of Kerman. In this study, the presence of CHV-1 in vaginal specimens and biopsies of the uterus of dogs referring to the Veterinary Hospital of Shahid Bahonar University of Kerman was determined. Samples were collected and evaluated using real-time PCR.

Results Viral DNA was detected in 21 samples from a total of 140 (15%) collected samples which were related to 14 uterine samples (20%) and 7 (10%) vaginal specimens. The association of this virus with age, breed, housing, pregnancy and reproductive disorders was not significant.

Conclusions This study is the first molecular detection of CHV-1 in reproductive samples of dogs in Iran. Considering the significant prevalence of this virus, it is necessary to carry out management measures in controlling and preventing this disease. Tracing CHV-1 requires further research on this virus in dogs of this region.

Background

Canine herpesvirus-1 (CHV-1), an alpha herpesvirus, is considered as a significant cause of neonatal deaths and reproductive problems in bitches with a widespread distribution [1-3]. In 1965, Carmichael et al. described this pathogen for the first time [4]. It has been well documented that the cell tissues of domestic and wild Canidae can only be infected by CHV-1 [5-7]. Virus is transmitted through exposure with secretions from respiratory or genital tracts of infected animals. Newborn puppies can be infected in utero, through the birth canal or contact with oronasal and genital mucosal secretions [4, 8, 9]. CHV-1 infection causes various clinical presentations based on the age of infected animals [5]. Newborn puppies are susceptible to manifest an acute fatal generalized systemic illness which is characterized by progressive multifocal hemorrhagic necrosis in multiple organs [3, 9, 10]. Mild subclinical infection or inapparent clinical illness is observed in puppies older than 3 weeks [11]. Adult domestic dogs can be clinically asymptomatic or display canine infectious respiratory disease complex and genital papulovesicular lesions [12]. Reproductive disorders including abortion, fetal death, mummification, stillbirth, premature or weak newborn puppies are observed in pregnant bitches [9, 10, 13]. Recently, CHV-1 was correlated to the ocular lesions including conjunctivitis and ulcerative and non-ulcerative keratitis [12]. Lifelong latent infection can be established after recovery [6, 12]. In stressful conditions such as overcrowding, transportation, pregnancy and immunosuppression, CHV-1 reactivates and replicates, and therefore reshedding occurs [12, 14, 15]. CHV-1 infection is diagnosed serologically by serum neutralization test (SNT) and enzyme linked immunosorbent assay (ELISA), virus isolation, fluorescent antibody techniques and electron microscopy. Polymerase chain reaction (PCR) is recognized as the most reliable method of

identifying latent infection in adult dogs. A real-time PCR assay has also been established to detect CHV-1 nucleic acid [5].

Although no vaccine is commercially available in Iran and serologic data point to the circulation of CHV-1 among dogs, no definitive diagnosis has been conducted based on the molecular assay. In a recent study from south-east of Iran, the seroprevalence of CHV-1 was reported to be 20.7% in dogs [16]. Various predisposing factors have been reported to influence the incidence of CHV-1, but the conclusions have been inconsistent. Considering the global spread of this virus and its economic importance due to reproductive disorders, this research was conducted to determine the prevalence of CHV-1 in dogs of Kerman by real-time PCR assay and to investigate the possible risk factors.

Results

In this study, a total of 140 reproductive samples were collected and submitted to real-time PCR targeting the *gB* gene to detect CHV-1. Viral DNA was detected in 21 out of 140 (15%) reproductive samples collected from the referred dogs. In uterine samples, 14 specimens (20%) were positive for viral DNA whereas CHV-1 was identified in 7 out of 70 (10%) vaginal samples (Table 2). Out of 14 positive uterine samples, 6 specimens were collected from adult dogs ≤ 2 years old, 7 samples were belonged to dogs between 2 to 7 years old and one case was above 7 years old age. Although, no statistically significant differences were observed between all groups ($P = 1$), the rate of positive samples was greater in dogs less than 7 years old age. Regarding vaginal samples, in the group of adult dogs, two dogs which were under 2 years old age were positive for CHV-1 DNA. Furthermore, viral DNA was amplified in three out of 37 cases aged between 2 to 7 years old and two of 13 dogs which were above 7 years old age. No association was identified between age and presence of CHV-1 viral DNA ($P = 0.9$). Distribution of CHV-1 positive samples according to the age groups of dogs is shown in table 3.

Viral DNA was identified from the uterine samples of six client-owned and eight shelter dogs. There was no statistically significant difference among all the groups which were classified according to the type of housing ($P = 0.7$). The presence of CHV-1 DNA was confirmed in vaginal samples of three client-owned and four stray dogs but no statistically significant differences was seen between groups ($P = 0.9$). Distribution of CHV-1 positive samples considering the type of housing of dogs is summarized in table 3.

Out of 14 CHV-1 positive uterine samples, three ones were belonged to dogs which were pregnant at the time of sampling while all of seven positive samples of vaginal specimens were included in group of the non-pregnant dogs. Nevertheless, there were no statistically significant differences between the groups ($P = 0.7$). Only two out of 14 positive uterine samples and three out of 7 positive vaginal specimens were belonged to the dogs with a history of reproductive disorders. Pyometra and metritis were diagnosed in two positive uterine samples and stillbirths, vaginitis and vaginal prolapse were observed in dogs with positive vaginal swabs. Nevertheless, these differences between the mentioned factors were not

statistically significant ($P = 0.7$). Association between detection of CHV-1 in reproductive tract samples of dogs and reproductive parameters is revealed in table 3.

Discussion

CHV-1 is an important and universal cause of neonatal mortalities and reproductive disorders [2, 17, 18], and canine serve as the only hosts for this virus. The fact that this pathogen can cause infertility shows the importance of this infection. So, veterinarians should warn breeders regarding the economic importance of this organism, especially in newborn puppies and pregnant bitches. This is the first study for molecular detection of CHV-1 in reproductive samples of dogs in Iran. According to the results of this study, it is determined that reproductive organs of bitches admitted to the Veterinary Teaching Hospital of Shahid Bahonar University of Kerman have been infected by CHV-1.

After first description of virus by Carmichael et al. (1965), numerous studies have demonstrated CHV-1 to be enzootic in different parts of the world [2, 15]. Serologic investigations in domestic dogs have been reported from 0 to as high as 100% in some areas [6]. Ronsse et al. (2002) reported an overall CHV-1 seroprevalence of 45.75% in the Belgian dog population. In another study reported in 2008, the prevalence of antibodies against CHV-1 in the serum of dogs was determined as 22% using SNT and ELISA in the Gauteng Province of South Africa [19]. Dahlbom et al. (2009) also reported 81.5% seroprevalence of CHV-1 in Finland [11]. Furthermore, the seroprevalence of CHV-1 infection was determined as 85.5% by immunoperoxidase monolayer assay (IPMA) in Norway [20]. Similarly, in a study performed in turkey in which virus neutralization test (VNT) and ELISA were used for investigation of CHV-1 seroprevalence, antibody against CHV-1 was detected in 39.3% of samples by ELISA in comparison to 29.4% of the specimens using VNT [21]. In this area, only one study conducted by Babaei et al. in 2010 and the overall CHV-1 seroprevalence was reported to be 20.7% by using indirect immunofluorescence antibody (IFA) assay. In comparison, CHV-1 DNA was detected in 21 out of 140 (15%) referred dogs using real-time PCR in the current study.

Compared to our results, other researchers have reported various prevalence of CHV-1 using molecular assays. Using real-time PCR technique, Losurdo reported that not only CHV-1 developed a fatal systemic illness clinically and histologically, but also it could result in long-term shedding of the virus through the nasal and ocular secretions and the faeces [3]. So, infected puppies pose a threat for other animals in the colony. Cargnelutti et al. (2015) also described an outbreak of systemic fatal illness causing neonatal mortalities using histopathology, VNT and PCR [22]. In study of Larsen et al. (2015) performed in Denmark, 13 out of 57 (22.8%) dead puppies were positive for CHV-1 infections by real-time PCR (qPCR) while histopathological and in situ hybridization results were inconsistent [23]. Moreover, CHV-1 was defined as a respiratory virus and detected in nasal swabs of dogs via PCR [24, 25]. Adult dogs also can be implicated in spread of infections. Reactivation of lifelong latent infection of adult dogs can be recognized after environmental stressors and CHV-1 reshedding occurs. Ledbetter et al. in 2009 proved that viral reactivation of latently infected adult dogs with CHV-1 can be induced following administration of an immunosuppressive dosage of systemic prednisolone. Similarly, Malone et al. (2010) described a case of

disseminated CHV-1 infection in an immunocompromised adult dog [26]. In the mentioned case, blood sample and vaginal swab were positive for CHV-1 by PCR indicating a viremia. Moreover, Gadsden et al. (2012) reported a case of fatal CHV-1 infection in a 9-year-old spayed female Bichon Frise dog which infection was diagnosed by PCR, immunohistochemistry, and in situ hybridization [27]. Surprisingly, the case had no history of immunosuppression. So, the author stated "CHV-1 should be included in the list of differential diagnoses of hepatic necrosis in adult dogs". In a study accomplished in Mexico, CHV-1 infection of dead puppies was confirmed using histopathology, direct immunofluorescence, electron microscopy and PCR [2].

To the knowledge of the authors, the present study is the first molecular detection of CHV-1 in reproductive samples in Iran. Particularly, there was no study regarding detection of viral DNA in uterine specimens in literature. In the present study, 14 out of 70 uterine samples (20%) were found positive for viral DNA while CHV-1 was detected in 7 out of 70 (10%) vaginal samples. In contrast to our findings, Ronsse et al. (2005) indicated that despite seroconversion, none of the vaginal and nasal swabs or buffy coats were confirmed positive for CHV-1 [13]. In the study of Pratelli et al. (2014) in Italy, an overall seroprevalence of 14.6% and 18.6% was determined using SNT and in-house immunofluorescence (IF), respectively; but CHV-1 DNA was not found in none of the vaginal swabs [18]. In another study conducted in Italy, none of the submitted sera were CHV-1 positive by SNT and nested PCR assays [7]. Li et al. in 2016 reported that replication and invasion of CHV-1 in vaginal mucosa is experimentally better than respiratory mucosa according to the latitude and penetration depth of the plaques of viral antigen positive cells [17]. It seems that the reproductive system is considered a better target rather than the respiratory system for virus.

Our finding is similar to other studies in which there was no statistical difference between age and detection of CHV-1 [7, 14, 19, 21]. In contrast to our findings, other researchers indicated that the rate of the infection was significantly higher in older dogs compared with younger ones [16, 25, 28]. We did not found breeds of dogs as a predisposing factor for infection. In accordance with our findings, other researchers also could not find any relation between CHV-1 and the breed of infected dogs [7, 19]. Despite insignificant difference in study of Yeşilbağ et al., prevalence of CHV-1 was higher in Golden Retrievers (56.2%), followed by Terriers (50.0%) in turkey [21]. Moreover, we did not find any significant difference between CHV-1 detection in privately owned and stray dogs in current study. In agreement with our findings, other investigators demonstrated that the CHV-1 prevalence is not necessarily associated with various housing types [8, 16, 18]. However, there have been a number of reports in which higher prevalence was observed in dog colonies rather than client-owned dogs [21].

Relation between CHV-1 infection and reproductive disorders has been established in previous studies. Dahlbom et al. (2009) found an association between the rates of antibody titers and reproductive problems [11]; although, other researchers demonstrated no significant relationship between CHV-1 infections and reproductive abnormalities [14, 18] which is in accordance with our study. In the study conducted by Ronsse et al., 2004, no considerable association was determined between reproductive conditions and CHV-1 antibody titers in spite of a tendency to more abortions in seropositive breeding kennels [13, 28]. There were no statistically significant differences among pregnancy status and CHV-1

infections in this study. Some authors also did not find any association between CHV1 antibody titers to reproductive parameters such as pregnancy [7, 14]. Ström Holst et al., 2012 indicated pregnancy presumably does not result in reactivation of the infection when there is good sanitary and managements [15]. Excretion of CHV-1 in the vaginal mucosa during whelping was considered as a risk factor for puppies by Kapil, (2015). In study of Ström Holst et al. in 2012, viral reactivation of latent CHV infection was evaluated during gestation on pregnancy consequence or in the course of non-pregnant luteal phase. For this purpose, twelve mated and eight control bitches were assessed by detection of antibody against CHV-1 in blood samples and viral DNA in vaginal swab using real-time PCR. Interestingly, any dependable difference in antibody titers was not observed in these stages and vaginal swab was not CHV-1 positive [15].

The prevalence of CHV-1 infection depends on the geographic regions, variation in sampling, and also the largely various study populations. The difference between sensitivity of the various diagnostic methods can also affect the results. CHV-1 is considered as a poorly immunogenic virus which antibodies against of it disappear during a few months after exposure [7, 28]. Moreover, serologic assays have not been standardized and different results can be extracted from various labs. This emphasizes that the seroprevalence of infection can be underestimated using serologic techniques. In the current study, real-time PCR was used to detect CHV-1 in reproductive samples. PCR is well known as one of the most reliable methods for detecting CHV-1 in puppies and latent infections of adult dogs [5, 6]. Kapil in 2015 optimized a PCR test for detection of CHV-1 DNA in formalin-fixed, paraffin embedded (FFPE) sections and stated "The *gB* gene offered a suitable PCR target for CHV-1 detection in FFPE samples".

Conclusion

Until now, this study is the first molecular detection of the presence of CHV-1 in Iran. According to the results of this study, reproductive samples of dogs can harbor CHV-1 and being exposed to this population and their excretions may lead to a higher prevalence of infection. These findings are in agreement with previous study conducted by Babaei et al. (2010) in southeast of Iran, which has demonstrated that CHV-1 infection is endemic in the dog population of Iran and could pose a huge threat for breeders regarding neonatal mortalities and reproductive problems. Since risk factors for infections have not been established, vaccination of bitches seems logical particularly in problematic kennels with valuable breeding dogs. To avoid outbreaks, early diagnosis and good management are necessary. Accordingly, this is advisable to reduce environmental stressors for the dam and puppies, to improve sanitary and nutritional conditions and to enhance temperature during 3 weeks after birth for newborn puppies [10]. Further epidemiologic researches are required for better understanding the distribution of this pathogen in Iran.

Methods

Sample collection and DNA extraction

Samples (N=140) were collected from female dogs presented to the Veterinary Teaching Hospital of Shahid Bahonar University of Kerman from April 2016 to October 2018. All following procedures were approved by the Animal Care Committee of Veterinary College of Shahid Bahonar University of Kerman (No: 950102). Also, all samples were obtained after getting permission from dogs' owners. Uterine samples were achieved from 70 female dogs including 47 shelter and 23 client-owned dogs that were referred for elective or therapeutic ovariohysterectomy surgery. Vaginal samples were also taken from 70 dogs comprising 31 stray and 39 pet animals which were not considered for surgery. For each animal, a detailed questionnaire was prepared to record predisposing factors including age, breed, type of housing (indoor or outdoor), health status, and history of pregnancy, whelping or reproductive disorders during sampling. Dogs belonging to the study group and risk factors predisposing to CHV-1 infection are shown in Table 1. CHV-1 vaccine was not administered for any of the dogs.

Vaginal swab samples were collected by sterile rayon tipped applicators (Puritan®, Maine, USA) and transferred into the sterile 1.5 ml microtubes containing 500 µl sterile phosphate buffered saline (PBS). For uterine samples, a full-thickness specimen (a 3×3 mm piece) was acquired from each dog during ovariohysterectomy surgery. The uterine specimens were also preserved in a sterile 1.5 ml microtube comprising 70% ethanol. DNA was extracted from specimens using the DNA extraction Mini Kit (GenAll®Exgene TM, Korea), according to the manufacturer's instruction and stored at -20 °C. A negative extraction control was included with every 3 samples.

Molecular detection of CHV-1 (Real-time PCR assay)

Real-time PCR was performed to screen the glycoprotein B (*gB*) gene as previously described (Decaro et al., 2010). Total volume of each PCR reaction was 20 µl containing 10 µl of RealQ Plus 2x Master Mix Green (Ampliqon, Denmark), 0.3 µl of each primer (work stock solution was 10 pmol/µl), 2.5 µl of DNA template and 6.9 µl of distilled water. In *gB*-positive samples, forward (5'- ACAGAGTTGATTGATAGAAGAGGTATG-3') and reverse (5'-CTGGTGTATTAACTTTGAAGGCTTTA-3') primers will result in amplicons of 439-465 bp. The PCR thermal conditions were 1 cycle at 95°C for 15 min (for activation of hot start Taq DNA polymerase) followed by 40 cycles including denaturation (95°C for 15 s), annealing (65°C for 20 s) and elongation (72°C for 20 s). Also melting step (95°C for 10 s, 65°C for 10 s and 97°C for 1 s) was performed for more analysis. Distilled water and DNA extracted from a slide of CHV-1 indirect immunofluorescence antibody (IFA) test kit (Mega Screen FLUO C.HV; MegaCor, Horbranz, Austria) were considered as negative and positive controls, respectively. After completion of the real-time PCR run, threshold cycle number (CT) and melting temperature (T_m) of each amplicon were automatically determined via a software on LightCycler 96® System (Roche, Germany).

Statistical analysis

Statistical analysis was carried out using SPSS software (version 21; SPSS Inc.) and p <0.05 was considered statistically significant.

Abbreviations

Canine herpesvirus-1 (CHV-1); Real-Time PCR: Real-Time Polymerase Chain Reaction

Polymerase Chain Reaction

Declarations

Ethics approval and consent to participate

All implementation phases of this study were approved by the Animal Care Committee of Veterinary College of Shahid Bahonar University of Kerman.

Consent for publication

Not applicable.

Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

M.R (Supervisor of Thesis) guided experimental design, wrote the manuscript and analyzed the data; M. J was the Advisor of Thesis and performed PCR of the experiment; R. A was involved in collecting uterine and vaginal samples of dogs and performing PCR; M. K (Supervisor of Thesis) guided experimental design and provided advice for PCR experiments; H. B was the Advisor of thesis and supplied the positive control. All authors read and approved the final manuscript.

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Tables

Table 1: the study groups of dogs and predisposing factors to CHV-1 infection

Sample	Age			Breed	Pregnancy		Reproductive disorders		Housing		Herpesvirus	
	≤ 2 years	2-7 years	≥7 years		Yes	No	Yes	No	Indoor	Outdoor	Yes	No
Dog (Uterine)	26	35	9	Crossbreed: 51, Terrier: 11, Rottweiler: 1, Doberman: 1, German: 2, Dachshund: 2, Samoyed: 1, Spitz: 1	15	55	Total: 13; Vaginal prolapse: 1, Pyometra: 3, Breast tumor: 3, Dystocia: 1, Metritis: 3, Uterine atresia: 1, Abortion: 1	57	23	47	14	56
Dog (Vagina)	15	40	15	Terrier: 18, Crossbreed: 17, Rottweiler: 1, German: 18, Dachshund: 2, Bulldog: 1, Husky: 1, Pitbull: 2, Boxer: 2, Great Dane: 1, Spitz: 5, Pomeranian: 1, Pointer: 1	7	63	Total: 20; Still birth: 4, Vaginal prolapse: 2, Pyometra: 7, Breast tumor: 3, Dystocia/ metritis: 1, Mating problem: 1, Vaginal disorders: 2	50	39	31	7	63

Table 2: Frequency and percentage of detected CHV-1

Samples	Frequency	Percentage (%)
Uterine (dog)	14 from 70	20
Vaginal (dog)	7 from 70	10
Total	21 from 140	15

Table 3: Association between detection of CHV-1 in reproductive tract samples of dogs and various factors

Variable/Sample	Uterine (dog)		Vagina (dog)	
	Positive/ Negative	P value	Positive/ Negative	P value
Age				
Under 2 years	6/20	1	2/13	0.979
Between 2 to 7 years	7/28		3/37	
Above 7 years	1/8		2/13	
Pregnancy	Positive/ Negative	P value	Positive/ Negative	P value
Yes	3/12	0.780	0/7	0.757
No	11/44		7/56	
Reproductive disorders	Positive/ Negative	P value	Positive/ Negative	P value
Yes	2/11	0.722	3/17	0.769
No	12/45		4/46	
Housing	Positive/ Negative	P value	Positive/ Negative	P value
Indoor	6/17	0.706	3/36	0.989
Outdoor	8/39		4/27	