Detection of Canine herpesvirus 1 in the breeding kennel and farm dogs by Real-time PCR

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Abstract

Background: Canine herpesvirus 1 (CHV-1) is known as a causal agent of death in newborn puppies and fertility problems in adults with a widespread distribution. There has been an increasing concern among dog breeders in Iran regarding CHV-1. This study is the first molecular detection of CHV-1 in breeding kennels and farm dogs in Iran using real-time PCR and investigation of various predisposing factors.

Results: A total of 63 vaginal samples collected from 47 breeding kennels and 16 farm dogs were evaluated. In general, 21 out of 63 (33.3%) of vaginal samples were CHV-1-positive. The percentage of infection was higher in farm dogs, which was statistically significant. There was no significant association regarding other probable predisposing factors, including age, breed, pregnancy, and reproductive disorders.

Conclusions: Considering the results of this study, CHV-1 is common in farm and breeding kennels and could pose a threat. Further studies are required to better understanding the distribution of the virus within Iran to advise dog breeders to more appropriate measures for management of CHV-1.

Background

Canine herpesvirus 1 (CHV-1) is an α –herpesvirus with a global distribution. This pathogen has got the attention of veterinarians and dog breeders due to reproductive disorders and neonatal mortalities all over the world [1–3]. The host range of CHV-1 is documented to be restricted to domestic and wild canids [1, 4, 5]. Virus transmission occurs through respiratory or genital routs [6, 7]. CHV-1 can be transmitted in utero, through the birth canal or contact with mucosal secretions to newborn puppies [6, 8]. Clinical manifestation can vary based on the age and immune status of the infected animals [4]. Neonatal puppies younger than 1 to 3 weeks are susceptible to develop a fatal multisystemic disease characterized by focal necrohemorrhagic disorders in parenchymatous organs [9, 10]. In puppies older than 3 to 4 weeks and adults, asymptomatic or mild and subclinical self-limiting infections are mostly diagnosed. The virus is responsible for fetal death, abortion, mummification, or stillbirth in pregnant bitches [11]. Moreover, respiratory disease, ocular signs, and genital lesions are also documented [5, 12–14]. Lifelong latent infection is associated with localization of CHV-1 to nerve ganglia following recovery [1]. By reactivation of virus, CHV-1 replicates, and viral shedding occurs in stressful conditions or following immunosuppression [5, 15, 16]. Various diagnostic methods have been established to detect CHV-1 infection. The polymerase chain reaction (PCR) is well known as the best technique especially for detecting latent infection [1, 4, 5].

Serologic studies of various parts of the world indicated an infection range between 0% to as high as 100% of domestic dogs in some kennels [1]. In this area, clinical reports compatible with CHV-1 infection have been commonly described, and serological data specify the persistence of CHV-1 among kennels [17]. Despite clinical relevance following its economic importance, no definitive diagnosis has yet been established. So, this study was designed to determine the persistence of CHV-1 in breeding kennels of dogs and animals kept in the farm in the southeast of Iran using real-time PCR and investigate possible predisposing factors to guide dog breeders for effective management.

Results

Sixty-three bitches from 5 breeding kennels and seven farms located in the southeast of Iran (Kerman city), which were accepted sampling included in this study. Vaginal swabs were collected and evaluated using real-time PCR for CHV-1 infection. In total, viral DNA was detected in 21 out of 63 (33.3%) vaginal specimens (Table 1). All of the dogs had not been vaccinated against CHV-1 infection previously. The frequency of dogs belonged to the study groups based on various factors is shown in Table 1.

Twenty out of 63 (31.7%) dogs had less than two years old, and 43 (68.3%) dogs were between 2 and 7 years old in the time of sampling (Table 1). PCR analysis detected ten dogs included in the group of lower than two years old was positive.

Additionally, in dogs between 2 and 7 years old age, 11 animals had been infected by CHV-1. There was no statistical association between the detection of CHV-1 infection and age (P= 0.29) (Table 3). In general, sampling was performed from 14 different breeds that the highest number was related to German shepherd dogs (66.7%) (Table 1). As illustrated in Table 3, any significant association was not found between breed and detection of CHV-1 (P= 0.77). Of six (9.5 %) dogs were pregnant in the time of sampling, three dogs were CHV-1-positive. Furthermore, reproductive disorders were diagnosed in 25 (39.7%) dogs that CHV-1 was found in 7 of them. No differences were observed between CHV-1-negative and CHV-1-positive animals considering to the pregnancy (P= 0.90). Furthermore, no statistical association was found regarding the histories and parameters of reproductive disorders of the bitches (P= 0.71) (Table 3). The frequency of dogs based on housing is illustrated in Table 1. Among 47 (74.6%) vaginal specimens collected from breeding bitches, 14 samples had positive results. A higher prevalence of infection was related to the dogs belonging to the Fouladi kennel (Table 2). Sixteen (25.4%) samples were collected from dogs kept in the farm in contact with other animals, including horse, sheep, and cow. Seven out of 16 samples belonged to the group of farm dogs had positive results (Table 2). The percentage of positive specimens was higher in the farm dogs (7 out of 9) rather than kennel/breeding dogs (14 out of 33). A significant association was observed between disease status and housing type (P = 0.00) (Table 3). Association between detection of viral DNA in vaginal samples and various factors is illustrated in Table 3.

Discussion

Up to now, the significant role of CHV-1 in the fertility and neonatal mortality has been precisely determined. Moreover, recent researches indicate this virus is enzootic in the dog population of numerous countries [9, 13]. The present survey is the first molecular study to detect CHV-1 in vaginal samples of the kennel and farm Iranian dogs. The results show that the majority of the kennel and farm bitches have been infected by CHV-1.

After the first recognition of CHV-1 by Carmichael et al. in 1965 [10], various studies have been done all around the world regarding this cytopathic pathogen. Krogenæs et al. (2014) reported a seroprevalence of 85.5% using immunoperoxidase monolayer assay (IPMA) in the Norwegian adult dog population [18]. Yeşilbağ et al. (2012) also investigated the Turkish dog population. They found a higher antibody prevalence (39.3%) by enzyme-linked immunosorbent assay (ELISA) compared with the virus neutralization test (VNT) (29.4%) with a difference statistically significant [19]. A research performed by Dahlbom et al. in 2009 showed that the seroprevalence of CHV-1 was 81.5% in Finnish breeding kennels with and without reproductive problems, which the higher antibody titers were related to the dogs with reproductive problems [20]. In another study accomplished in South Africa, the sera of 328 dogs from 38 breeding kennels were evaluated using serum neutralization test (SNT), and ELISA, and 22% of sera were reported positive for the antibody of CHV-1 [2]. Only one study has been performed in the southeast of Iran that used indirect immunofluorescence antibody (IFA) assay [17]. In the mentioned study, a total seroprevalence was presented as 20.7%, which was 22.9% and 19.1% for kenneled and owned dogs, respectively [17]. In comparison, CHV-1 was detected in 21 out of 63 (33.3%) vaginal samples using real-time PCR in the current study, which positive specimens were contributed to the 50% and 77.7% kennel and farm dogs respectively.

In the present study, vaginal samples were collected and evaluated using PCR. Selection of vaginal specimens was made according to an experimental research accomplished by Li et al. in 2016, which shown a better capacity of replication and invasion in vaginal mucosa rather than respiratory mucosa [21]. Serological techniques are not a good choice to detect CHV-1 infections due to poorly immunogenic characteristics of the virus, non-standardized and invalidated tests, and variation in the level of positivity among different laboratories [1]. This emphasizes that PCR is the best technique to detect active and latent CHV-1 infections [1, 4, 5]. In the study of Lara et al. conducted in 2016, CHV-1 infections were confirmed in dead puppies using histopathological evaluation, direct immunofluorescence, electron microscopy, and PCR for DNA polymerase and glycoprotein B genes [22]. The author could find an association between the macroscopic and microscopic findings of necropsied pups and the presence of CHV-1 DNA [22]. Decaro et al., 2010 also described a real-time PCR as a useful tool to confirm a clinical diagnosis, to study viral pathogenesis, and to evaluate the efficacy of vaccines and antiviral

drugs [4]. Moreover, Kapil (2015) also optimized a CHV-1–specific PCR targeting gB gene to detect DNA in formalin-fixed, paraffin-embedded (FFPE) tissues and offered gB gene as an appropriate target [23].

Researches in which molecular assays were used to detect CHV-1 in various samples were little. Infection with CHV-1 was determined using VNT and PCR in puppies presenting dyspnea, pale mucous membranes, abdominal pain, and finally death by Cargnelutti et al. in 2015 [9]. These cases showed focal hemorrhage and necrosis of parenchymatous organs such as the liver and kidneys [9]. Another study performed in Denmark by Larsen et al. (2015) presented that CHV-1 was found in 22.8% of tissue pools of the dead puppies using real-time PCR (qPCR), however histopathological and in situ hybridization findings were inconsistent [24]. Moreover, CHV-1 infection associated with respiratory diseases was detected in 32 out of 100 nasal and oropharyngeal swabs of dogs and 6 out of 32 pleural effusions using multiplex PCR in Thailand [14]. In another study conducted in Brazil, CHV-1, as a respiratory virus, was found in 6% of the nasal secretions of shelter dogs via PCR [25]. In contrast, other studies were not identified CHV-1 infections using molecular assays. Ronsse et al. (2005) assessed 27 breeding bitches during one reproductive cycle. Despite seroconversion, CHV-1 DNA was not found in any of the vaginal and nasal samples or buffy coats [11]. Moreover, in the study of Pratelli et al. (2014), any vaginal swabs were not positive for CHV-1, while an overall seroprevalence of 14.6% and 18.6% were determined using SNT and IFA respectively [26]. Bottinelli et al. (2016) could not find CHV-1 infection in a dog breeding kennel using SNT and nested PCR assays [15].

In the current study, similar to previous researches, detection of CHV-1 was independent of age [2, 15, 27]. On the contrary, CHV-1 infection was significantly higher in dogs older than three years old compared with younger ones [17]. An increase of CHV-1 antibody titer with age was also reported by Ronsse et al. (2004) [7]. Fourteen different breeds were evaluated for CHV-1 in the present study, but any significant association was not found between breed and status of diseases. This finding was similar to the study of Bottinelli et al. in 2016 [15]. In comparison, Yeşilbağ et al. in 2012 determine the highest CHV-1 prevalence in Golden Retrievers (56.2%), followed by Terriers (50.0%) without any significant association [19].

CHV-1 can establish latent infection in adults, which reactivates following immunosuppression. Ledbetter et al. (2009) describe viral reactivation and ocular disease recrudescence after administering the systemic prednisolone to adult dogs latently infected with CHV-1 [5]. Malone et al. (2010) also reported a case of disseminated CHV-1 infection in an immunocompromised adult dog which infection was confirmed via PCR CHV-1-positive of vaginal and blood specimens [28]. On the contrary, a case of fatal CHV-1 infection in a 9-year-old spayed female Bichon Frise dog with no history of immunosuppression was reported in another study [8]. It has well been known that stressful conditions such as pregnancy result in viral reactivation and shedding and subsequently, neonatal infections [1]. In this study, 6 out of 63 (9.5%) dogs were pregnant in the time of sampling that half of these samples were positive. Still, there was not any statistical association between pregnancy status and CHV-1 infection. Similarly, Ström Holst et al. in 2012 could not detect any dependable variation in antibody titers in pregnant and non-pregnant phases [3]. Ström Holst et al., 2012 also stated, "pregnancy alone does not seem to cause reactivation of the infection when management is good, and other stressing factors are absent" [3]. Moreover, Krogenæs et al., 2012 determined no association between CHV1 antibody titers and reproductive parameters like pregnancies [27].

In the current study, reproductive disorders were established in 25 (39.7%) dogs that 7 out of these 25 dogs were infected with CHV-1. However, no significant association was identified regarding reproductive disorders. Interestingly, only one dog had vaginal papules consistent with CHV-1 infection in the vaginal examination at the time of sampling. Fouladi kennel had a history of stillbirth, abortion, and neonatal mortality and showed the highest prevalence in breeding dogs. In agreement with our findings, other researchers could not determine any significant differences in CHV-1 infections between bitches with reproductive disorders compared with healthy fertile dogs [7, 11, 26]. Ronsse et al., 2004 also demonstrated a tendency towards serological results and a history of abortion in bitches [7]. According to Krogenæs et al., 2012, no significant associations was observed between titer of antibody and breeding status, including the history of mattings, whelping, infertility, and conditions of puppies [27]. On the contrary, Dahlbom et al., 2009 observed significant higher CHV-1 titers in dogs from kennels with reproductive problems rather than kennels with no history of reproductive disorders [20].

In this study, the percentage of positive specimens was higher in the farm dogs (7 out of 9) compared with the kennel dogs (14 out of 33). The association between detection of CHV-1 and housing type was highly significant. It is in agreement with Yeşilbağ et al., who also determined a higher CHV-1 seroprevalence in the colony dogs (62.1%) compared with the individual dogs (39.0%) [19]. In contrast, other researchers could not find any differences between privately owned dogs and kenneled dogs. Raising status (owned or kennels) was not perceived as a predisposing factor in the study of Babaei et al. in 2010 [17]. Pratelli et al., 2014 also described no association between disease status and housing type [26]. Colony size, management, and hygienic conditions can influence the incidence of CHV-1 infection via the reactivation of latent infections and subsequent viral spread [20]. Sampling during fall and winter months when the kennel ambient temperature is set below 21 °C, predisposes newborn puppies to CHV-1 due to hypothermia [23]. In the present study, dogs from 5 breeding kennels and seven farms in Iran were sampled during fall and winter months. In the kennels, most dogs were housed individually in a cage, but sometimes during the day, the animals were being kept outdoors in fenced areas to run and exercise; so, the dogs of varying ages had contact with each other. The hygienic and dietary conditions were not suitable for all of the times, and the dogs were fed once daily. Also, dogs were occasionally kept in farm households in contact with other animals, usually including sheep, cow, and horses. Poor sanitary and nutritional conditions were also seen. The difference between the detection of CHV-1 in various kennels and farms may be attributed to diverse hygienic and dietary requirements and the dog crowding/density of the husbandry. Due to reactivation and reinfection, there is a persistent CHV-1 infection and its consequence on adults and newborn puppies.

Neonatal mortality is considered as an unavoidable problem in breeding kennels ranged between 5–35% [29–31]. Despite significant importance and economic loss, the mortality of puppies either in parturition time or after that is poorly documented. According to the stillborn and dead neonatal puppies, CHV-1 seems to be common in Iran, but its significance as a pathogenic cause of neonatal death remains unclear. Since dog breeders did not submit dead puppies for necropsy, histopathological examination and other diagnostic methods to determine and confirm the agent. So, CHV-1 infection expected to be underdiagnosed. Kapil, 2015 demonstrated the shedding of CHV-1 in the vaginal canal during parturition as a risk factor for newborn puppies [23]. Long-term shedding of CHV-1 in neonatal puppies was documented by Losurdo et al. in 2018 [13]. Immunosuppression following administration of systemic prednisolone to the latently infected adult dogs can also reactive the virus [5].

Conclusions

Considering the results of this study, it is demonstrated that CHV-1 is present in the southeast of Iran. CHV-1 is common in farm and breeding kennels and could pose a threat. Further studies are required to better understanding the distribution of the virus within Iran to advise dog breeders to more appropriate measures of management of CHV-1. In problematic kennels, vaccination against CHV-1 is justifiable, particularly in the valuable breeding stocks. Poor sanitary and nutritional conditions and overcrowding should be avoided. Eradication of CHV-1 from infected kennels is often impossible. So, breeders should diminish environmental stressors. Additionally, due to the temperature-sensitive characteristic of the virus, newborn puppies should be kept in warm places.

Methods

Sample collection and DNA extraction

In the present study, a total of 63 bitches, including 47 dogs from five breeding kennels and 16 farm dogs, were evaluated during 2017–2018. All study procedures were approved by the Animal Care Committee of Veterinary College of Shahid Bahonar University of Kerman (No: 970102). These kennels and farms which participated in this research, are located in various parts of Kerman city. Also, all samples were achieved after getting permission from dogs' owners. A detailed questionnaire was prepared to record the time of sampling, region, signalment, housing, the status of vaccination and deworming, pregnancy, whelping and the status of puppies, reproductive problems, the number of dogs, and hygiene

condition of enclosures. Then, a vaginal examination was performed to evaluate abnormal discharge, masses, and appearance of vaginal mucosa for CHV-1 accompanying lesions. For each animal, a vaginal swab was collected by sterile rayon tipped applicators (Puritan®, Maine, USA) and reserved in sterile 1.5-ml Eppendorf tube containing phosphate buffer saline (PBS). All specimens were stored at -80 C for further analysis. The extraction of DNA from vaginal swabs was done using a Viral Nucleic Acid Extraction Kit (Roche, Germany) according to the manufacturer's instructions. A negative extraction control was considered between every three samples to screen the nucleic acid extraction efficiency.

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

In this study, Real-time PCR targeting the glycoprotein B (gB) gene was accomplished. A total volume of 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 was submitted for each PCR reaction. In gB-positive samples, forward (5'- ACAGAGTTGATTGATAGAAGAGGTATG-3') and reverse (5'-CTGGTGTATTAAACTTTGAAGGCTTTA-3') primers will produce amplicons of 136 bp. The PCR thermal cycling consisted of one cycle at 95ºC for 15 min for activation of hot start Taq DNA polymerase followed by 40 cycles, including denaturation at 95ºC for 15 s, annealing at 65ºC for 20 s, and elongation at 72ºC for 20 s. Also, the melting step at 95ºC for 10 s, 65ºC for 10 s, and 97ºC for 1 s was also considered for more analysis. In each assay, distilled water and DNA extracted from a slide of CHV-1 indirect immunofluorescence antibody (IFA) test kit (Mega Screen FLUO CHV; MegaCor, Horbranz, Austria) were included as negative and positive controls, respectively. After completion of the real-time PCR run, threshold cycle number (CT) and melting temperature (Tm) of each amplicon were automatically analyzed using software on LightCycler 96® System (Roche, Germany).

Statistical analysis

Statistical analysis was achieved using SPSS software (version 21; SPSS Inc.) by the generalized linear model (GLM), and $p < 0.05$ was determined statistically significant.

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; S. K 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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Abbreviations

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

Polymerase Chain Reaction

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Tables

Table 1: The study groups of kennel and farm dogs and predisposing factors to CHV-1 infection

	Age			Breed	Housing		Pregnancy			Reproductive disorders		Herpesvirus	
ginal ples)	< 2 years	2-7 years	≥7 years	Type and number of breeds	Kennel	Farm	Yes	No	Estrus and mating	Yes	No	Yes	No
uency	20	45	0	German shepherd: 42; Rottweiler: 1; Malinois: 1; Qafqaz: 1; Sarabi: 3; Great dane: 1; Qahdarijani: 1; Alabai: 2; Spitz: 1; Chihuahua: 2; Mastiff: 4; Terrier: 2; Crossbreed: 1; Husky: 1	47 Fouladi Kennel 1: 22; Khiz Kennel: 13; Behzadi Kennel: 6; Fouladi Kennel 2: 1; Qazizadeh Kennel: 5	16 Khiz Farm (Horse): 2; Saeed Farm (Horse): 8; Faramarzpour Farm (Cow): 1; Shaheidri Farm (Sheep): 2; Haji Razmpour (Sheep): 1; Afzali Farm (Sheep): 1; Sistani Farm (Sheep): 1	6	50	7	Total: 25; Still birth: 3; Vaginal disorders: 1; Pyometra: 1; Death of newborn puppies: 9; Mating problem: 5; Infertility: 5; Pseudopregnancy: 1	38	21	42
rcent %)	31.7	68.3	0	German shepherd: 66.7; Rottweiler: 1.6; Malinois: 1.6; Qafqaz: 1.6; Sarabi: 4.8; Great dane: 1.6; Qahdarijani: 1.6; Alabai: 3.2; Spitz: 1.6; Chihuahua: 3.2; Mastiff: 6.3; Terrier: 3.2; Crossbreed: 1.6; Husky: 1.6	74.6 Fouladi Kennel 1: 34.9; Khiz Kennel: 20.6; Behzadi Kennel: 9.5; Fouladi Kennel 2: 1.6; Qazizadeh Kennel: 7.9	25.4 Khiz Farm (Horse): 3.2; Saeed Farm (Horse): 12.7; Faramarzpour Farm (Cow): 1.6; Shaheidri Farm (Sheep): 3.2; Haji Razmpour (Sheep): 1.6; Afzali Farm (Sheep): 1.6; Sistani Farm (Sheep): 1.6	9.5	79.4	11.1	Total: 39.7 Still birth: 4.7; Vaginal disorders: 1.5; Pyometra: 1.5; Death of newborn puppies: 14.2; Mating problem: 7.9; Infertility: 7.9; Pseudopregnancy: 1.5	60.3	33.3	66.7

Table 2: Association between dogs belonging to the study group (kennel and farm dogs) and detection of CHV-1 in vaginal samples

Housing	Name	Herpes		
		Positive	Negative	Total
Kennel	Fouladi Kennel 1	10	12	22
	Khiz Kennel	1	12	13
	Behzadi Kennel	2	4	6
	Fouladi Kennel 2	1	0	1
	Qazizadeh Kennel	0	5	5
Farm	Khiz Farm (Horse)	1	1	2
	Saeed Farm (Horse)	1	7	8
	Faramarzpour Farm (Cow)	0	1	1
	Shaheidri Farm (Sheep)	2	0	2
	Haji Razmpour (Sheep)	1	0	1
	Afzali Farm (Sheep)	1	0	1
	Sistani Farm (Sheep)	1	0	1
Total		21	42	63

Table 3: Association between detection of CHV-1 in vaginal samples of kennel and farm dogs and various factors

Variable/Sample	Vaginal samples (dog)		
Age	Positive	Negative	P value
Under 2 years	10	10	0.29
Between 2 to 7 years	11	32	
Above 7 years	0	0	
Breed	Positive	Negative	P value
German shepherd	15	27	0.77
Rottweiler	0	1	
Malinois	0	1	
Qafqaz	0	1	
Sarabi	1	2	
Great dane	0	1	
Qahdarijani	0	1	
Alabai	0	2	
Spitz	0	1	
Chihuahua	0	2	
Mastiff	2	2	
Terrier	1	1	
Crossbreed	1	0	
Husky	1	0	
Pregnancy	Positive	Negative	P value
Yes	3	3	0.90
No	15	35	
Estrus and mating	3	4	
Reproductive disorders	Positive	Negative	P value
Yes	7	18	0.71
No	14	24	
Housing	Positive	Negative	P value
Kennel	14	33	0.00
Farm	7	9	