Scientific Research Publishing

Open Journal of Veterinary Medicine, 2016, 6, 149-162
http://www.scirp.org/journal/ojvm
ISSN Online: 2165-3364
ISSN Print: 2165-3356

Canine Herpesvirus Seroprevalence and Associated Factors in Dogs of Mexico

Edgar Guillermo Valdivia Lara[1], Jesica Ileana Ángeles Solis[1], Cesar Cuenca Verde[1], Juan Antonio Montaraz Crespo[2], Laura Cobos Marín[3], Juan Carlos Del Río García[4], Guillermo Valdivia Anda[1*]

[1]Laboratorio de Patogenicidad Microbiana, Unidad de Investigación Multidisciplinaria Facultad de Estudios Superiores Cuautitlán, Universidad Nacional Autónoma de México, Cuautitlan Izcalli, México
[2]Laboratorio de Inmunología, Facultad de Estudios Superiores Cuautitlán, Universidad Nacional Autónoma de México, Ciudad de México, México
[3]Facultad de Medicina Veterinaria, Universidad Nacional Autónoma de México Ciudad Universitaria, Ciudad de México, México
[4]Laboratorio de Micotoxinas, Unidad de Investigación Multidisciplinaria, Facultad de Estudios Superiores Cuautitlán, Universidad Nacional Autónoma de México, Ciudad de México, México
Email: guillermo897@hotmail.com, jessypezy@hotmail.com, ccuencaverde@hotmail.com, jamc1591@gmail.com, laura.cobosmarin@gmail.com, mcjcrg@gmail.com, *valdivag@unam.mx

How to cite this paper: Lara, E.G.V., Solis, J.I.Á., Verde, C.C., Crespo, J.A.M., Marín, L.C., Del Río García, J.C. and Anda, G.V. (2016) Canine Herpesvirus Seroprevalence and Associated Factors in Dogs of Mexico. *Open Journal of Veterinary Medicine*, **6**, 149-162.
http://dx.doi.org/10.4236/ojvm.2016.610019

Received: August 15, 2016
Accepted: October 24, 2016
Published: October 27, 2016

Abstract

Canine herpesvirus (CHV-1) causes disease associated with high mortality in infected puppies, which represents large financial losses for dog breeders. Since CHV-1 at the time of the study he had not been reported in Mexico, the main objective of this study was to determine the prevalence of antibodies against CHV-1 in canine kennels in the metropolitan area of Mexico City. A commercial enzyme-linked immunosorbent assay (ELISA) was used, and the results were compared to those of a viral neutralization test. The ELISA kit uses the complete viral particle as the antigen. The plaque reduction neutralization test was combined with the immunoperoxidase technique because of the low cytopathic effect of CHV-1. Neutralizing antibodies were also detected in 20 randomly selected samples. The prevalence of CHV-1 with ELISA was 87%. The concordance between ELISA and serum neutralization (SN) was 0.1129, the sensitivity of the ELISA against SN was 1.0 (100%), the positive predictive value was 0.39 (39%), and the negative predictive value was 1 (100%). These results show that ELISA is useful for monitoring the dog population for CHV-1; a positive test result requires confirmation with an SN test, and a negative ELISA result indicates a high probability of being SN-negative. The only variables that were statistically associated with CHV-1 prevalence were breed and kennel. A statistically significant relationship between the degree of ELISA and SN titer was obtained, with a confidence level of 95%. None of the clinical presentation factors was statistically significant. These results suggest that most of the canine population studied in Mexico is in a herpesvirus latency state.

Keywords

Canine Herpesvirus Seroprevalence, Enzyme-Linked Immunosorbent Assay, Serum Neutralization Test

1. Introduction

There are currently only one reports of herpesvirus disease in dogs in Mexico, and the disease is not found on any of the lists issued by the International Organization for Animal Health (OIE). However, canine herpesvirus type I (CHV-1) has been isolated in various countries worldwide, and recent studies in Europe suggest that the disease is present in the canine population [1]-[4]. CHV-1 is associated with high mortality in infected puppies, which represents large financial losses for dog breeders. Because of the lack of evidence of its prevalence in Mexico, there are currently no available options for diagnosis, treatment, or prophylaxis of CHV-1 infection. It is considered that the virus is poorly immunogenic, and generates neutralizing antibodies that disappear within only a few months after infection [5].

Recent studies have estimated the worldwide population seroprevalence of CHV-1 at 30% - 40%, varying according to the country that reports, with a higher prevalence in canine kennels, considering the size of the farm, hygiene, and kennel cough as potential risk factors. A relationship between seroprevalence, history of abortions, and neonatal mortality has also been established [4] [6]. These results have all been based on detection of immunoglobulin G (IgG) antibodies by enzyme-linked immunosorbent assay (ELISA). Furthermore, inhibition of lytic plaques and reduction in fluorescent spots have been employed in the technical serum neutralization (SN) technique has been mainly used for detection of canine herpesvirus and varicella-zoster virus (VZV, belonging to the same viral genus) antibodies [7]-[11]. Canine herpesvirus, like other members of the genus *Varicellovirus*, usually remains strongly attached to the cell, resulting in very weak cytolytic effects. Two passages of the viral isolates previously obtained in the laboratory [12] were conducted in the chicken embryo by inoculation at the chorioallantoic membrane. This is a widely used technique for replication of other herpesvirus species such as herpes simplex virus (HSV)-1, HSV-2, HSV-3, VZV, laryngotracheitis virus, and pseudorabies [13]. However, to date, there have been no reports of canine herpesvirus replication in the chicken embryo.

The objectives of the present study were to determine the prevalence by the detection of specific IgG antibodies against CHV-1 in canine kennel breeding centers of the metropolitan area of Mexico City, using a commercial ELISA test, to compare the results of ELISA with those of the viral neutralization test, and to determine factors related to the medical history of the animals with the presence of antibodies.

2. Materials and Methods

A serological survey was performed in 8 kennels distributed 1 in Mexico City, 3 in the

State of Mexico, 2 Queretaro and 2 in the city of Guadalajara Jalisco in a period from August 2014 to February 2016. To calculate the required sample size, we adopted previously reported statistical methods [12], the sample size calculation is done considering that the prevalence in the country is unknown and that the objective was to compare two diagnostic tests [13]. A sample size of 96.04 individuals was obtained through this method, and therefore we decided to use 100 dogs. Inclusion criteria were: canines inhabiting the kennel, over 1 year and under 7 years of age, and clinically healthy at the time of sampling, were excluded animals showing any clinical disease, animals less than 1 year and animals in which the medical history of the last 3 months was unaware. Three to five milliliters of Blood was collected without anticoagulant, using Vacutainer™ tubes, from the saphenous or jugular vein in and centrifuged at 1200 ×*g*, for 10 min to room temperature in a clinical centrifuge to obtain serum. The serum was dispensed into Eppendorf tubes and frozen at −70˚C until use. The number of animals that were allowed to sample per kennel was: Mexico City (8), 3 in the State of Mexico (13, 32 and 3), Queretaro (11 and 7) and in the city of Guadalajara Jalisco (22 and 4). The Kennels and breeding distribution they are shown in **Table 1**. The kennels were selected from the database of customers that counts to particular veterinary diagnostic laboratory, DIVeT™, in which there was a history of possible illness, owners were asked for their participation and animals that met the inclusion criteria were sampled randomly. All sampled kennels are breeders breeding pure breeds and often are carried to dog shows on the country.

A survey, questionnaire, was performed during sampling in which the following data were collected: kennel, animal, and history of diseases associated with herpesvirus infection in dogs [4] such as reproductive problems, eye secretions , skin injury, and respiratory secretions.

A commercial ELISA kit (EVL™ Woerden, the Netherlands) was used to detect the presence of antibodies against CHV-1. This kit uses the complete viral particle as the antigen. The samples were processed according to the manufacturer recommendations by preparing 1:30, 1:90, 1:270, and 1:810 dilutions of the positive and negative controls and a 1:250 dilution of the serum sample. After the procedure was completed, the absorbance of the wells was read at 450 nm in a Multiskan Gen, (Thermo Scientific™) reader. Calculation of the titer was performed based on the results of the absorbance (calculated titer) of each sample, with the assumption that the positive control has a titer of 3600, as indicated by the manufacturer of the kit (EVL™ Woerden).

The data were processed using Statgraphics® software, with generalized linear models, taking the calculated titer as the dependent variable and all the following factors as independent variables: age, sex, breed, kennel, weight, height, country of birth, if the dog had copulated or not, the number of litters, and history of respiratory, eye, skin, or reproductive conditions.

Because the virus used by us at work has a very low cytolytic effect [14], we employ a combination of routine test SN and observe cytopathic effect of the virus by the detection of cell infection by an immunoperoxidase technique, using antibodies against ca-

Table 1. Calculated titer of anti-CHV-1 (EVL™ ELISA) of animals sampled.

Sample identification	Kennel	Breed	Sex	Age (months)	Calculated titer
001	1	Pug	Female	39	*1069.4
002	1	Pug	Male	46	*295.5
003	1	Pug	Female	14	*376.1
004	1	Pug	Female	14	*854.1
005	1	Pug	Male	27	*1812.3
006	1	Pug	Male	13	*1492.2
007	1	Pug	Female	58	*462.2
008	1	Pug	Female	51	*545.0
009	1	Pug	Female	32	73.6
010	1	Pug	Female	37	−255.4
011	1	Pug	Female	24	*1000.9
012	2	Doberman	Male	13	*1120.2
013	2	Bulldog	Female	42	*760.3
014	3	German shepherd	Female	48	−204.6
015	3	German shepherd	Male	36	−252.1
016	3	German shepherd	Female	72	−483.9
017	3	German shepherd	Female	36	−382.4
018	3	German shepherd	Female	14	*166.3
019	3	Westy	Female	84	*2049.7
020	3	Westy	Female	24	*351.8
021	3	Westy	Female	29	*1864.2
022	3	Westy	Female	24	*225.9
023	3	Westy	Female	29	*2748.5
024	3	German shepherd	Female	60	−336.0
025	6	Neapolitan mastiff	Female	48	*521.8
026	6	Neapolitan mastiff	Macho	45	*1390.6
027	7	German shepherd	Female	39	*240.3
028	7	German shepherd	Female	30	*242.5
029	7	Westy	Male	72	−63.3
030	7	Westy	Female	48	*570.4
031	6	Neapolitan mastiff	Female	48	*2037.6
032	2	Great Danes	Female	18	*1494.4
033	2	Great Danes	Female	30	*2190.9
034	3	German shepherd	Female	42	*140.2
035	7	German shepherd	Male	72	*337.8

Continued

036	7	German shepherd	Male	24	*950.4
037	7	German shepherd	Female	30	*1996.7
038	4	Great Danes	Male	12	*2083.9
039	4	Great Dane	Male	12	*963.2
040	4	Great Dane	Male	13	*221.5
041	4	Great Dane	Female	12	*1366.6
042	4	Great Dane	Female	84	*1021.4
043	4	Great Dane	Female	48	*1956.0
044	4	Great Dane	Female	48	*3262.7
045	4	Great Dane	Male	60	*2718.7
046	5	Dachshund	Female	72	*1200.4
047	5	Dachshund	Female	83	*766.8
048	5	Dachshund	Female	83	*972.5
049	5	Dachshund	Male	36	*967.9
050	5	Dachshund	Female	38	*1449.2
051	5	Dachshund	Male	84	*452.9
052	5	Dachshund	Male	60	*1103.9
053	5	Dachshund	Female	13	*1306.2
054	5	Dachshund	Female	12	*1355.0
055	5	Dachshund	Female	84	*2655.9
056	5	Dachshund	Female	38	*1037.6
057	5	Dachshund	Female	83	*1052.7
058	5	Dachshund	Male	12	*1530.5
059	5	Dachshund	Male	81	*605.2
060	5	Dachshund	Female	25	*1915.3
061	5	Dachshund	Male	84	*667.9
062	5	Dachshund	Female	36	*1398.0
063	5	Dachshund	Male	82	*198.3
064	5	Dachshund	Male	48	*264.5
065	5	Dachshund	Female	84	*1670.1
066	5	Dachshund	Male	83	*2361.8
067	5	Doberman	Female	24	*869.3
068	5	Doberman	Female	25	*1253.4
069	5	Doberman	Male	80	*1283.6
070	5	Doberman	Female	12	*1176.8
071	5	Doberman	Male	13	*714.9
072	5	Doberman	Female	13	*886.7

Continued

073	5	Doberman	Male	13	*1276.6
074	5	Doberman	Male	13	*218.2
075	5	Golden Retriever	Female	36	*834.5
076	5	Doberman	Male	60	*1333.5
077	8	Dachshund	Female	36	*887.8
078	8	Dachshund	Female	12	*981.8
079	8	Dachshund	Female	12	*484.0
080	8	Dachshund	Male	14	*1347.4
081	8	Dachshund	Female	36	12.8
082	8	Dachshund	Female	12	*130.0
083	8	Dachshund	Male	36	*2575.3
084	8	Dachshund	Female	66	*1786.1
085	8	Dachshund	Female	84	*732.3
086	8	Dachshund	Female	84	*1807.0
087	8	Dachshund	Female	72	*3812.5
088	8	Golden Retriever	Male	30	*550.1
089	8	Chihuahua	Female	60	−142.8
090	8	Chihuahua	Female	84	61.5
091	8	Chihuahua	Female	16	*423.6
092	8	Chihuahua	Female	84	25.5
093	8	Dachshund	Female	12	*1580.7
094	8	Dachshund	Female	80	*125.3
095	8	Dachshund	Male	48	*651.1
096	8	Dachshund	Female	72	*880.9
097	8	Dachshund	Female	84	*1308.0
098	8	Dachshund	Male	12	*1182.6
099	5	Doberman	Female	48	*779.9
100	3	Chihuahua	Female	62	−95.2

*Positive result.

nine herpes virus of dog and anti canine IgG subsequently antibodies conjugated to peroxidase (Sigma™) developed by diaminobenzidine (Sigma™) on wells with cells [7]. Neutralizing antibodies were tested in 20 randomly selected samples

A plaque formation test was used for viral titration. Ten-fold dilutions of the virus isolate (1:10, 1:100, 1:1000, 1:10,000) were made, and 0.5 ml of each was added to each well of a 12-well NUNC™ plate containing a monolayer of Madin-Darby canine kidney MDCK cells (In vitro™, p117) at 90% confluence in minimal essential medium (MEM) (In vitro™) with 5% newborn calf serum (NCS) (In vitro™). The cells were incubated for

1 h at 35˚C to allow for viral absorption, with mixing every 15 min. Finally, MEM was added slowly with 1% newborn calf serum and 0.6% agarose (Bioline™) at 45˚C to allow for solidification, and then incubated at 35˚C for 3 days. A drop of formaldehyde (JTBaker™) was then added to each well so that the agarose could be subsequently removed. The wells were washed carefully with phosphate buffered saline (PBS) and fixed with acetone/methanol (ICR™) for 10 min. Subsequently, 0.5 ml of a 1:80 dilution of positive anti-CHV-1 control serum (EVL™) was added to each well and incubated for 30 min at 37˚C. The wells were washed three times with PBS, and anti-canine IgG peroxidase (Sigma Aldrich™) was added and incubated for a further 30 min at 37˚C. The plate was washed three times with PBS, and a 0.01% diaminobenzidine solution (Sigma Aldrich™) with hydrogen peroxide (ICR™) was added and held at 15 min at 37˚C The plate was washed again with PBS and stained with Harris hematoxylin (Sigma Aldrich™) for 15 s, and the plates were examined microscopically for the presence of plaque forming units, termed peroxidase-positive spotlights (PPS), which were counted only in wells containing 20 - 100 plaques (Figure 1).

The neutralization assay was performed by incubating equal amounts of virus (50 PPS) with sera at different dilutions (1:4, 1:8, 1:16, 1:32, and 1:64; pretreated for 30 min at 56˚C) for 30 min at 37˚C. The mixture (0.5 ml) was inoculated to NUNC™ plates containing a monolayer of MDCK cells at 90% confluence. Viral adsorption was allowed for 1 h at 35˚C, while redistributing the inoculum every 15 min, and MEM supplemented with 1% calf neonate serum and 0.6% agarose at 45˚C was added slowly, allowing for solidification. The plate was incubated at 35˚C for three days, and then a drop of 40% formaldehyde was added to each well and the agarose layer was removed the next day. The titer was calculated as the inverse of the highest dilution in which a reduction of at least 50% of the number of plaques was observed. A serum sample was considered negative if the 1:8 dilution of serum did not show a decrease of 50% or more in plaque formation.

Simple regression analysis was used to determine the correlation between the results

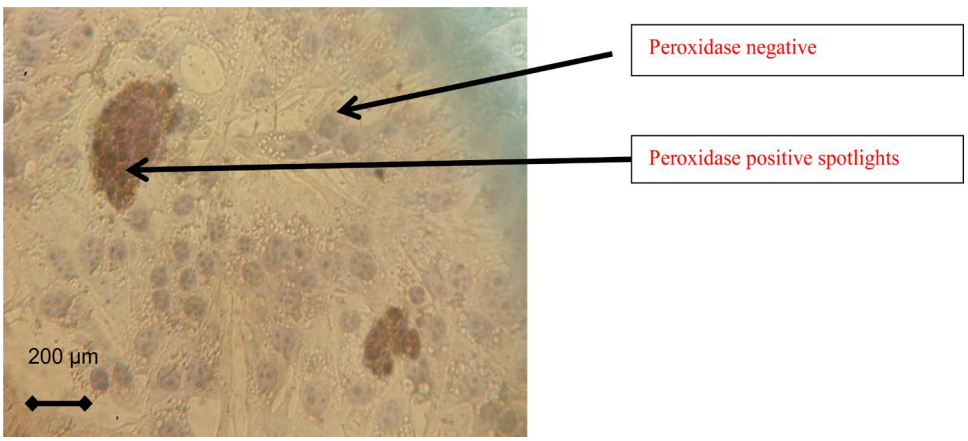

Figure 1. Example of the results taken as positive in the methodology peroxidase positive spotlights (PPS) on cell culture. MDCK cells were infected with canine herpesvirus and incubated for 72 h, and then stained with peroxidase and diaminobenzidine. Magnification, 400×.

calculated by ELISA and those obtained from the SN test. For the construction of the model, titers under 8 in the SN and an ELISA result < 100 were considered negative (value of 0) according to published by Gerna, and Chambers [7], for Varicella.

3. Results and Discussion

The majority of the dogs sampled were females 68% (68/100); the average age was 35.43 months (2.95 years) the standard deviation was 25.7. Overall, 86% of the individuals were born in Mexico, and the remaining 7 were born in the USA (1), Chile (4), Argentina (1), Denmark (1), and Spain (1). Most (85%) of individuals had never left the country, and 61% of the sampled animals had mated, with 32 of the 68 females having produced at least one litter. According to the survey, 26% of the sampled individuals had presented reproductive problems, 14% had a history of infectious tracheobronchitis, 1% had eye infections, and 4% had skin diseases (unrelated to the virus cutaneous presentation). Conventional vaccination schedule was achieved in all the dogs, and none had been previously immunized against CHV-1.

Figure 2 shows the simple linear regression graph of the ELISA results, including the regression equation, slope, and intercept. According to the regression equation, the titer was calculated using the absorbance (Table 1). Some of the calculated titers were negative because of the equation, not the actual observation. Besides, the titles relate to some of the analyzed clinical parameters (Table 2).

According to the personal recommendations of Dr. Van Herwijnen (EVL™ Woerden, the Netherlands), a result was considered positive if the calculated titer exceeded 100. Of the 100 samples tested, 87 were positive

The plaque formation test showed a viral titer of 1.72×10^4 PFU/ml viral culture. Viral neutralization results were performed for 20 samples from canines selected randomly (Table 3). Besides we related to some of the analyzed clinical parameters with ELISA and Serum neutralization test (Table 4).

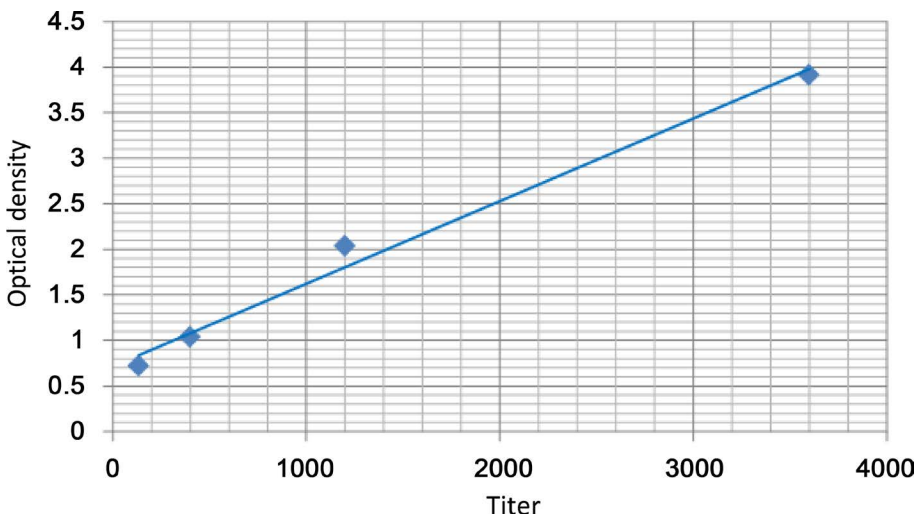

Figure 2. Relationship of the optical density obtained in the ELISA test (EVL™ Woerden, the Netherlands) with the positive control serum titer: $y = 0.0009x + 0.7173$. $R^2 = 0.9924$.

Table 2. Relationship between clinical parameters and results of the ELISA test (EVL™ ELISA).

Parameter	Variable	Elisa negative	Elisa positive
Kennel	1	2 (18.2%)	9 (81.8%)
	2	0 (0%)	4 (100%)
	3	6 (46.2%)	7 (53.8%)
	4	0 (0%)	8 (100%)
	5	0 (0%)	32 (100%)
	6	0 (0%)	3 (100%)
	7	1 (14.3%)	6 (85.7%)
	8	4 (18.2%)	18 (81.8%)
Breed	E. Bulldog	0 (0%)	1 (100%)
	Chihuahua	4 (80%)	1 (20%)
	G. Retriever	0 (0%)	1 (100%)
	Dachshund	1 (2.6%)	37 (97.4%)
	Doberman	0 (0%)	11 (100%)
	Great Dane	0 (0%)	10 (100%)
	L. Retriever	0 (0%)	1 (100%)
	Neapolitan M.	0 (0%)	3 (100%)
	German Shep.	5 (41.7%)	7 (58.3%)
	Pug	2 (18.2%)	9 (81.8%)
	West highland WT	1 (14.3%)	6 (85.7%)
Sex	Male	2 (6.3%)	30 (93.75%)
	Female	11 (16.2%)	57 (83.8%)
Age	Young (24 months old or less)	0 (0%)	30 (100%)
	Adult (more than 24 months old)	13 (18.6%)	57 (81.4 %)
Total		**13 (13%)**	**87 (87%)**

The results for Simple regression analysis are shown in the Figure 3. The overall concordance between the ELISA and SN results was 0.1129, which is considered to be low [15], this can be explained, since although both tests measure the levels of antibodies, the detection methods differ. The SN technique detects only neutralizing antibodies that interfere with viral infection directly, whereas the ELISA is unable to distinguish between neutralizing and non-neutralizing antibodies, and therefore has a broader antibody repertoire. In addition, the ELISA detects antibodies that cross reactive with other proteins, as the test used works with the complete virus but only detects IgG antibodies. Moreover, canine herpesvirus is only weakly immunogenic, and antibodies specific to the virus remain at high titers in the blood for only a short window [11] [16]; therefore, the SN assay might fail to detect low levels of antibodies.

The sensitivity of the ELISA test with respect to the SN result was 1 (100%). In 1989, Takumi *et al.* [9] developed the indirect ELISA technique, and detected a 26.2% positivity rate in contrast to a 5% positive rate obtained by the neutralization technique in 557 dogs. Therefore, ELISA is considered a more sensitive and practical test [17]. Such a

highly sensitive diagnostic test is useful in clinical settings where the failure to diagnose a disease (*i.e.*, false negative) would create more problems than overdiagnosis (*i.e.*, false positives). This is the case of a screening test, which is performed by applying a diagnostic test that gives valid and reliable results that are inexpensive, easy to perform, and causes minimal discomfort to the patient.

In this study, the positive predictive value was 39%, indicating that a sample that is deemed positive by the ELISA test has only a 39% chance to be positive by the SN test. This value is relatively low, suggesting that a positive result obtained in a clinical setting or epidemiological study would have to be confirmed by the reference test (SN). Moreover, the negative predictive value was 100%, indicating that if a sample is negative on the ELISA test, it has a 100% chance to be negative in the reference test (SN). This value indicates that a negative result on ELISA can be considered a true negative with confidence.

In the general linear model, breed and kennel were the only variables statistically as-

Table 3. Serum neutralization results and ELISA titers in 20 randomly selected dogs.

Sample identification	Kennel	Breed	Sex	Age (months)	Calculated titer (ELISA)	Titer (serum neutralization)
002	1	Pug	Male	46	295.5	64
015	3	German shepherd	Male	36	−252.1	<8
016	3	German shepherd	Female	72	−483.9	<8
018	3	German shepherd	Female	14	166.3	<8
022	3	Westy	Female	24	225.9	<8
025	6	Neapolitan mastiff	Female	48	521.8	<8
031	6	Neapolitan mastiff	Female	48	2037.6	8
032	2	Great Dane	Female	18	1494.4	<8
039	4	Great Dane	Male	12	963.2	<8
043	4	Great Dane	Female	48	1956.0	64
045	4	Great Dane	Male	60	2718.7	32
048	5	Dachshund	Female	83	972.5	16
051	5	Dachshund	Male	84	452.9	32
063	5	Dachshund	Male	82	198.3	<8
066	5	Dachshund	Male	83	2361.8	64
071	5	Doberman	Male	13	714.9	<8
079	8	Dachshund	Female	12	484	<8
084	8	Dachshund	Female	66	1786.1	<8
091	8	Chihuahua	Female	16	423.6	<8
094	8	Dachshund	Female	80	125.3	<8

ELISA titers were calculated and the serum neutralization was scored as the reciprocal of the highest dilution that inhibited 50% plaque formation.

Table 4. Agreement between clinical parameters, the ELISA and serum neutralization test.

Parameter	Variable	Elisa test		Serum titer neutralization		Agreement between the two tests
Kennel	1	Positive	1	Positive	1	100%
		Negative	0	Negative	0	100%
	2	Positive	1	Positive	0*	0%
		Negative	0	Negative	1	0%
	3	Positive	2	Positive	0	0%
		Negative	2	Negative	4	50%
	4	Positive	3	Positive	2	66.67%
		Negative	0	Negative	1	0%
	5	Positive	5	Positive	3	60%
		Negative	0	Negative	2	0%
	6	Positive	2	Positive	1	50%
		Negative	0	Negative	1	0%
	8	Positive	4	Positive	0	0%
		Negative	0	Negative	4	0%
Breed	Chihuahua	Positive	1	Positive	0	0%
		Negative	0	Negative	1	0%
	Dachshund	Positive	7	Positive	3	42.85%
		Negative	0	Negative	4	0%
	Doberman	Positive	1	Positive	0	0%
		Negative	0	Negative	1	0%
	Great Dane	Positive	4	Positive	2	50%
		Negative	0	Negative	2	0%
	Neapolitan M.	Positive	2	Positive	1	50%
		Negative	0	Negative	1	0%
	German Shep.	Positive	1	Positive	0	0%
		Negative	2	Negative	3	66.67%
	Pug	Positive	1	Positive	1	100%
		Negative	0	Negative	0	100%
	West Highland WT	Positive	1	Positive	0	0%
		Negative	0	Negative	1	0%
Sex	Female	Positive	11	Positive	3	27.27%
		Negative	1	Negative	9	11.11%
	Male	Positive	7	Positive	4	57.14%
		Negative	1	Negative	4	25%
Age	Young (24 months old or less)	Positive	7	Positive	0	0%
		Negative	0	Negative	7	0%
	Adult (more than 24 months old)	Positive	11	Positive	7	63.64%
		Negative	2	Negative	6	33.33%
Total		Positive	18	Positive	7	38.89%
		Negative	2	Negative	13	15.38%

*For 100 animals tested by ELISA, only serum neutralization test is performed on 20 randomly selected animals.

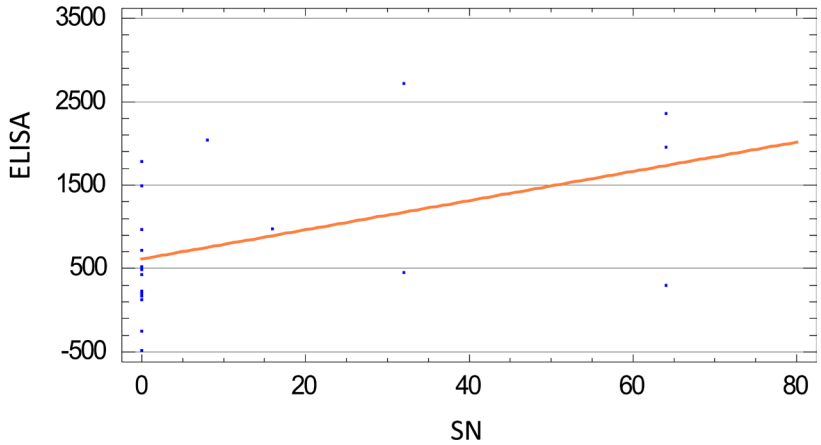

Figure 3. Linear regression of the titers obtained by enzyme-linked immunosorbent assay (ELISA) and the serum neutralization (SN) test. ELISA titers were calculated and SN results were scored as the reciprocal of the highest dilution that inhibited 50% of plaque formation. $Y = 612.6 + 17.54 \times X$; $R^2 = 21.40\%$.

sociated with the calculated titer, with a p-value of 0.0006 and R^2 of 0.3649, indicating that 36.49% of the variability in the calculated titer could be explained by these two variables. None of the factors of the clinical presentation such as infectious tracheobronchitis, skin or eye conditions, or reproductive problems had a statistically significant relationship with the serologically calculated titer, which suggests that most of the canine population studied is in viral latency or that the clinical presentations are due to other etiologies [18].

4. Conclusions and Implications

The high prevalence of CHV-1 disease in adult dogs in the central area of Mexico is highly significant when considering that in some areas, our previous research [14] showed that puppies from 1 to 3 months of age were killed by infection, and we were able to isolate strains of the virus. Although the litters belonged to different bitches from those sampled in the present study, our results confirm that canine herpesvirus is present in Mexico, causing clinical infection in newborn puppies and is present in latent form in adults, as has been found in other countries. Therefore, Mexico should adopt epidemic control tactics against the disease.

Overall, these results show that the ELISA test is useful for monitoring and screening of the canine population for CHV-1. A positive test result requires confirmation with the SN test, whereas a negative result in ELISA indicates a high probability of being SN-negative, and further confirmation is not required. Therefore, the ELISA test and SN test developed herein can be used to screen for viruses that have a very low cytolytic effect and are more highly associated with the cells by latency or integration of viral DNA into chromosome cell with or without cell transformation because the literature reports that despite being in viral latency, the body is able to form antibodies against the virus, so it is possible to detect by sensitive tests [16] [17]. This approach is expected to dramatically bring down the cost and time of diagnosis.

Acknowledgements

This work was supported by the Project PAPIIT-UNAM IT-202114 "Assessment of canine herpesvirus disease development in Mexico and possible measures for control and prevention".

References

[1] Lacheretz, A. and Cognard, S. (1998) Epidemiology and Serological Diagnosis of 10 Canine Herpesvirus. *Revue de Médecine Vétérinaire*, **143**, 1477-1488.

[2] Reading, M.J. and Field, H.J. (1998) A Serological Study of Canine Herpesvirus-1 Infection in the English Dog Population. *Archives of Virology*, **143**, 1477-1488. http://dx.doi.org/10.1007/s007050050391

[3] Rijsewijk, F.A.M., Luiten, E.J., Daus, F.J., Van der Heijden, R.W. and Van Oirschot, J. (1999) Prevalence of Antibodies against Canine Herpesvirus 1 in Dogs in the Netherlands in 1997-1998. *Veterinary Microbiology*, **65**, 1-7. http://dx.doi.org/10.1016/S0378-1135(98)00285-5

[4] Ronsse, V., Verstegen, J., Onclin, K., Friederic, F. and Poulet, H. (2004) Risk Factors and Reproductive Disorders Associated with Canine Herpesvirus-1 (CHV-1). *Theriogenology*, **61**, 619-636. http://dx.doi.org/10.1016/S0093-691X(03)00249-8

[5] Greene, C.E. (2012) Canine Herpesvirus Infection. In: Greene, C.E., Ed., *Infectious Diseases of the Dog and Cat*, 4th Edition, Saunders, Philadelphia, 48-54.

[6] Guigal, P.M., Fontbonne, A., Buff, S., Vincetti, M., Thévenet, F., Pavlowiez, S., *et al.* (2002) Prevalence of Antibodies against Canine Herpes Virus in French Breeding Kennels. *Proceedings of the* 3*rd EVSSAR European Congress on Reproduction in Companion*, *Exotic and Laboratory Animals*, Liege, 10-12 May 2002, 132; Cited in *Theriogenology*, **57**.

[7] Gerna, G. and Chambers, R. (1976) Varicella-Zoster Plaque Assay and Plaque Reduction Neutralization Test by the Immunoperoxidase Technique. *Journal of Clinical Microbiology*, **4**, 437-442.

[8] Engels, M., Mayr-Bibrack, B., Ruckstuhl, B. and Metzler, A. (1980) Seroepizootiology of Canine Herpesvirus in Switzerland and Preliminary Trials of a Vaccine. *Zentralblatt für Veterinärmedizin Reihe B*, **27**, 257-267. http://dx.doi.org/10.1111/j.1439-0450.1980.tb01691.x

[9] Takumi A., Kusanagi, K., Tuchiya, K., Xuan, X., Azetaka, M. (1989) Serodiagnosis of Canine Herpesvirus infection—Development of an Enzyme-Linked Immunosorbent Assay and Its Comparison with Two Improved Methods of Serum Neutralization Test. *Japanese Journal of Veterinary Science*, **52**, 241-250. http://dx.doi.org/10.1292/jvms1939.52.241

[10] Xuan, X., Horimoto, T., Limcumpao, J.A., Takumi, A., Tohya, Y., Takahashi, E. and Mikami, T. (1991) Neutralizing Determinants of Canine Herpesvirus as Defined by Monoclonal Antibodies. *Archives of Virology*, **116**, 185-195. http://dx.doi.org/10.1007/BF01319241

[11] Nöthling, J.O., Hössy, D., Steckler, D. and Ackermann, M. (2008) Seroprevalence of Canine Herpesvirus in Breeding Kennels in the Gauteng Province of South Africa. *Theriogenology*, **69**, 276-282. http://dx.doi.org/10.1016/j.theriogenology.2007.09.022

[12] Tilaki, K.H. (2014) Sample Size Estiamation in Diagnostic Test Studies of Biomedical Informatics, Methodological Review. *Journal of Biomedical Informatics*, **48**, 193-204. http://dx.doi.org/10.1016/j.jbi.2014.02.013

[13] Bell, M.L., Pinto, A.T., McKenzie, J.E. and Olivier, J. (2014) A Myriad of Methods: Calculated Simple Size for Two Proportions Was Dependent on the Choice of Simple Size For-

mula and Software. *Journal of Clinical Epidemiology*, **67**, 601-605.
http://dx.doi.org/10.1016/j.jclinepi.2013.10.008

[14] Valdivia-Lara, E.G., Barrón-Romero, B.L., Cobos-Marín, L., Ángeles-Solis, J.I., González-Gallardo S., Cuenca-Verde, C., *et al.* (2016) Pathology Isolation and Identification of Canine Herpesvirus (CHV-1) in Mexico. *Open Journal of Pathology*, **6**, 111-121.

[15] Watson, P.E. and Petrie, A. (2010) Method Agreement Analysis: A review of Correct Methodology. *Theriogenology*, **73**, 1167-1179.
http://dx.doi.org/10.1016/j.theriogenology.2010.01.003

[16] Carmichael, L.E. (1970) Herpesvirus Canis: Aspects of Pathogenesis and Immune Response. *Journal of the American Veterinary Medical Association*, **156**, 1714-1721.

[17] Ronsse, V., Verstegen, J., Onclin, K., Guiot, A.L., Aeberlé, C. and Nauwynck, H.J. (2002) Seroprevalence of Canine Herpesvirus-1 in the Belgian Dog population in 1997-1998. *Reproduction in Domestic Animals*, **37**, 299-304.
http://dx.doi.org/10.1046/j.1439-0531.2002.00363.x

[18] Carmichael, L.E. and Grene, C.E. (1998) Canine Herpesvirus Infection. In: Greene, C.E., Ed., *Infectious Diseases of the Dog and Cat*, WB Saunders, Philadelphia, 28-32.