

Short Paper

Studies on hemagglutination activity of *caprine herpesvirus-1*

Sirakov, I. N. * and Peshev, R. D.

National Diagnostic and Research Veterinary Medical Institute, 15 "Pencho Slaveykov" Blvd., 1606 Sofia, Bulgaria

*Correspondence: I. N. Sirakov, National Diagnostic and Research Veterinary Medical Institute, 15 "Pencho Slaveykov" Blvd., 1606 Sofia, Bulgaria. E-mail: insirakov@gmail.com

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Summary

The aim of this study was to investigate the hemagglutination properties of *caprine herpesvirus-1* (CpHV-1) and their application for CpHV-1 antigen detection and evaluation of the dynamics of post-infectious/vaccine antibody production. CpHV-1 hemagglutination activity was proved only with mouse erythrocytes, PBS and saline solutions at 4°C and 22°C. In vaccinated animals, goats and bucks with respiratory and genital clinical symptoms the antibody titers obtained by microtiter virus neutralization test (MVNT) were significantly higher ($P < 0.05$) compared to those determined by inhibition hemagglutination test (IHA), while in aborted animals the former were lower than the latter. The diagnostic specificity (Dsp) and sensitivity (Dse) of IHA was determined by comparison of the antibody titers obtained by MVNT. Between groups comparison of MVNT versus IHA test showed significantly higher ($\chi^2 = 8$, $P < 0.0047$) Dse of IHA for aborted animals and vaccinated animals only. No significant differences were found for the Dsp of any of the investigated groups. IHA test is a suitable method for detecting antibody production after the onset of the disease when IgM antibodies predominate and can be successfully used as an additional method for epidemiological studies and tracing of CpHV-1 antibodies formation and exhaustion.

Key words: *Caprine herpesvirus-1*, Hemagglutination, Mouse erythrocytes, Sensitivity

Introduction

Caprine herpesvirus-1 (CpHV-1) causes enteritis, acute generalized disease and mortality in 1- to 2-week-old kids, and, in goats, respiratory symptoms, vulvovaginitis, infertility, abortion, loss of newborn and growing kids. Infection affects the respiratory and genital tract in bucks causing balanoposthitis, short or long-lasting disruption of the mucosal surfaces, impossibility for breeding and disturbed spermatogenesis resulting in repeated insemination. The infection is most frequently subclinical or with weak clinical symptoms (Saito *et al.*, 1974; Horner *et al.*, 1982; Buonavoglia *et al.*, 1996).

The diagnosis is based on viral cultivation in cell cultures, electron microscopy, PCR and RT-PCR (Roperto *et*

al., 2000; Elia *et al.*, 2008), Microtiter virus neutralization test (MVNT), and enzyme linked immunosorbent assay (ELISA) have been used for sera testing (Marinaro *et al.*, 2010). As there is no CpHV-1 commercial ELISA, gB and gE BHV-1 ELISA have been used for serological detection of antibodies against CpHV-1 (Thiry *et al.*, 2008) based on the close antigenic relationship between those two viruses (Ros and Belak, 1999). The hemagglutination activity (HA) of CpHV-1 has been unsuccessfully investigated with bovine and guinea pig erythrocytes (Saito *et al.*, 1974; Horner *et al.*, 1982).

The aim of this study was to investigate: 1) the HA of CpHV-1, 2) the application of inhibition of hemagglutination (IHA) for diagnosis and 3) the dynamics of production and exhausting of CpHV-1 antibodies.

Materials and Methods

Dextrose gelatin veronal buffer (DGV); phosphate buffered saline (PBS); normal saline (NS), NS with Tween 80; borate buffer (BB) and carbonate buffer (CB), all with different pH were prepared (Payment and Trudel, 1993). Lyophilized bovine serum albumin 0.2% (w/v) was added to eliminate unspecific adsorption.

Human blood group O, bovine, sheep, goat, guinea pig, rabbit, bird, dog, horse, swine, rat, and mouse erythrocytes were used. Erythrocytes were washed three times with NS at 2000 rpm for 10 min.

Reference CpHV-1 strains were E/CH, McKercher, Spain 1, and Spain 2. Bovine corona virus and Kableskovo strain of mucosal disease viral diarrhea were used as heterologous viruses. Four Bulgarian CpHV-1 isolates-Troyan (Lab. Ref. No. 71/2006), Suhindol (Lab. Ref. No. 10/2007), Kyustendil (Lab. Ref. No. 107/2009) and Pavel Banya (Lab. Ref. No. 50/2007) were tested.

Viruses were cultivated on primary rabbit kidney (RK); Madin Darby bovine kidney (MDBK, CCLV 1992, RIE 261); Embryonic bovine trachea (EBTr ATCC NBL-4); Bovine esophagus (COP R, CCLV, RIE 244) cell lines. Cell cultures were grown on Sigma MEM Eagle with Hank's salt and L-Glutamine 0.2 M/L, 5% fetal calf serum (FCS), Penicillin 100 UI/ml, and Streptomycin 100 µg/mL. The same medium with 3% FCS was used for maintenance. After the total cytopathic effect the viruses were frozen and thawed three times and used. Reference strain E/CH was treated with polyethylene glycol 6000 (PEG 6000) to a concentration of 7% (Payment and Trudel, 1993).

Hemagglutination test (HAT) for antigen detection was performed according to Dilovski *et al.* (1982) and was read after complete settling in controls containing erythrocytes at 4°, 22° and 37°C.

Specificity of HAT was determined by hemagglutination inhibition test (HIT). Assayed antigen was diluted two-fold with NS, pH 6.9 in two rows of wells. In the first row 25 µL of NS were pipetted into each well. In the second row 25 µL of positive serum with antibodies against CpHV-1

(MVNT titer of 8 log₂ and 1:4 dilutions) were added. After incubation for 60 min at 4°C erythrocytes were added. Controls of positive antigen, positive serum, and erythrocytes were included. The reaction was read when agglutination was found in the positive antigen control and was considered specific when the difference between HAT and HIT was equal to or higher than 2 log₂.

Inhibition of hemagglutination (IHA) for detection of antibodies against CpHV-1 was performed for the serological investigations (Peshev *et al.*, 1989). The highest serum dilution causing complete inhibition of erythrocyte hemagglutination was taken as the antibody titer. A two-fold and higher increase of antibody titers in sera obtained 14-21 days after disease onset or vaccination were indicated as active virus infection or impact of vaccine virus.

To remove nonspecific virus inhibitors 40 serum samples were processed (Mengeling, 1972) and, for comparative study, were temperature treated at 56°C for 30 min.

The research included 103 blood samples obtained from goats with abortions, 27 from goats and bucks with respiratory and genital clinical symptoms, and 54 from vaccinated animals.

MVNT performed with CpHV-1 reference strain E/CH (titer 10^{7.5} TCID₅₀/mL) was used as a gold standard. Blood samples tested by MVNT and IHA were used for comparison.

Descriptive statistics were applied for all experimental variables. All continuous data (titers) were analysed for normality by the Kolmogorov-Smirnov test for determination of the appropriate statistical methods. Difference between groups for all parameters was assessed by nonparametric Mann-Whitney u-test. Wilcoxon matched pairs test was used for comparison of variables between different experimental conditions (MVNT and IHA test). The level of significance was set at P≤0.05.

Diagnostic sensitivity (Dse), diagnostic specificity (Dsp) and total agreement (TA) between MVNT and IHA were determined by equations of Samad *et al.* (1994).

Differences within and between the investigated groups of goats were compared

by 2×2 contingency tables with χ^2 -test set at 95% confidence interval and critical probability of 0.05.

Results

The highest virus hemagglutination titer was obtained with MDBK and EBTr cell lines (Table 1).

Table 1: HAT titers of reference strains and new CpHV-1 isolates after using different cell cultures

Viral strain/isolates	Cell cultures			
	RK	COP R	MDBK	EBTr
	HAT titers [\log_2]			
E/CH	3	2	6	5
McKercher	2	1	3	6
Spain 1	3	1	4	6
Spain 2	3	1	2	6
Tpoyan	3	3	5	6
Suhindol	3	1	6	5
Kyustendil	3	1	6	5
Pavel Banya	3	1	7	6

HA assay with CpHV-1 was only observed with 0.5, 0.75 and 1% mouse erythrocytes. The most suitable buffer systems were PBS and NS with pH = 6.9-7.2 (Table 2). At 37°C elution of erythrocytes was visible after 30 min and the viral titers were 1-2 \log_2 lower than those at 4°C.

Table 2: HAT titers of reference E/CH strain after using different buffers and temperatures

Buffers	pH	Temperature		
		4°C	22°C	37°C
		HAT titers [\log_2]		
DGVB	6.9	0	1	2
	7.2	0	1	2
PBS	6.9	3	2	2
	7.2	3	2	2
NS + Tween 80	6.9	3	2	2
	7.2	3	1	1
BB	6.9	lyses	lyses	lyses
	7.2	lyses	lyses	lyses
CB	6.9	lyses	lyses	lyses
	7.2	lyses	lyses	lyses
NS	6.9	4	3	2
	7.2	3	3	1

There was no significant increase ($P > 0.05$) of hemagglutination titers obtained after virus concentration with PEG 6000 and triple virus freezing and thawing. After concentration with PEG 6000 the HA titers reached 4 \log_2 and some of the viral

products were with a prosone effect up to 2 \log_2 .

The titers between HA and IHA were decreased with 2 \log_2 , and more when IHA was performed with CpHV-1 positive serum, indicating that the tested hemagglutination agent belonged specifically to CpHV-1.

After treatment of 40 serum samples by the method of Mengeling (1972) and temperature treatment only, no statistically significant differences were observed.

Significantly higher mean values of the antibody titer were observed by MVNT than by IHA test in the clinical symptoms group ($P < 0.05$, Wilcoxon matched pairs test) and the vaccinated animals ($P = 0.02$). On the contrary, in the group of aborted goats a significantly ($P = 0.01$, Wilcoxon matched pairs test) higher mean value of the antibody titer was obtained by IHA test compared to MVNT (Fig. 1).

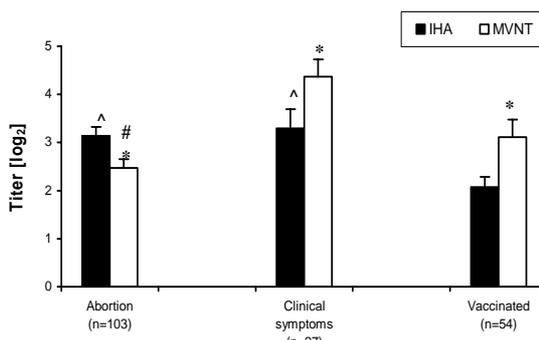


Fig. 1: Mean geometrical titers ± standard error of mean in serum samples investigated by MVNR and IHA tests. Statistically significant differences within a group are marked with * and between groups with ^ for IHA test or # for MVNT

The mean value of the antibody IHA titer was significantly higher ($P = 0.001$, Mann-Whitney u-test) in the group of aborted animals compared to the vaccinated animals, as well as for the animals with clinical symptoms than for the vaccinated animals ($P = 0.006$). It also revealed that the mean value of the antibody MVNT titer was significantly lower ($P = 0.001$) for the group of aborted animals compared to the group with clinical symptoms and vaccinated animals ($P = 0.003$, Mann-Whitney u-test).

The calculated Dse of the IHA test in aborted animals was 88.46%; Dsp, 40.00%;

and the total agreement between the two reactions, 76.69%. In animals with clinical symptoms these values were 86.96, 25.00 and 77.77% respectively, and for vaccinated animals - 71.79%, 33.33% and 61.11%, respectively.

Between groups comparison of MVNT with the IHA test showed significantly higher ($\chi^2 = 8$, $P=0.0047$) Dse of IHA for aborted animals and vaccinated animals only. The total agreement was significantly ($\chi^2 = 5.98$, $P<0.0144$) higher (76.69%) for aborted animals than that for vaccinated animals (61.11%) and between animals with clinical symptoms (77.77%) ($\chi^2 = 6.82$, $P<0.009$) and vaccinated animals. There were significant differences for Dsp only between the groups of the aborted and the clinical symptoms animals ($\chi^2 = 5.13$, $P<0.0235$).

Discussion

The hemagglutination activity of herpesviruses has been poorly studied and the results are heterogeneous. Trybala *et al.* (1993) have established that glycoprotein C (gC) is the main factor for HA of *herpes simplex virus-1* (HSV-1) and hypothesized that the mechanism of gC interaction with the heparan sulfate receptors on erythrocytes is the same for all herpesviruses. Like *swine herpesvirus 1* (Tetsu *et al.*, 1989) and HSV-1 (Trybala *et al.*, 1993), CpHV-1 showed HA with mouse erythrocytes only. Most probably the cause is the complex and heterogeneous macromolecule of heparan sulfate (Gallagher *et al.*, 1986) and its different rate of sulfating (Lycke *et al.*, 1991). Another explanation could be the specific spacing of anionic sulfate groups (Trybala *et al.*, 1993), which can influence the interaction with the virus.

The most probable reason for the prozone effect observed after treatment of the viral probes with PEG 6000 could be the heterogeneity of CpHV-1 hemagglutinins containing components which suppress hemagglutination at lower dilutions. After triple freeze-thawing this effect was not observed due to the changed proteins physical environment after freezing, causing changes in protein conformation (Bhatnagar *et al.*, 2007; Weska *et al.*, 2009).

The differences in HA titers of the isolates were possibly due to the low conservativity of the gC gene (Engels *et al.*, 1992). The most suitable cell cultures for preparation of antigen were MDBK and EBTr cells, which stimulate the formation of hemagglutinins to a greater extent.

The interaction of specific antibodies in the sera with viral hemagglutinins gC and the formation of antigen-serum complex, which does not allow the erythrocytes to agglutinate in the HA test, could explain the decrease in viral titers with two and more logarithms in IHA.

Temperature treatment was sufficient to remove the nonspecific inhibitors, as there were no significant differences between the two treatment methods for 40 serum samples.

The significantly higher antibody titers obtained by MVNT in vaccinated animals and in animals with clinical symptoms compared to those determined by IHA are most probably due to formation of neutralization antibodies. The antibody MVNT titers were lower than the IHA ones in aborted animals, most probably because of the recent infection and formation of IgM antibodies.

It can be concluded that, although the sensitivity of the IHA test is lower compared to that of MVNT, IHA can be successfully used for early serological diagnosis of the disease, when IgM antibodies predominate. In this respect the IHA test can be applied as an additional method, together with MVNT, for epidemiological studies and tracing of the formation and exhaustion of different post-vaccination antibody classes.

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