LETTER TO THE EDITOR

STUDY OF BOVINE HERPES VIRUS 1 SPREADING AMONG BUFFALO HERDS IN BULGARIA

R. PESHEV[1*], L. CHRISTOVA[2]

[1]Central Veterinary Research Institute, 15a P. Slaveikov Blvd., 1606 Sofia, Bulgaria; [2]Institute of Biophysics, Bulgarian Academy of Sciences, Sofia, Bulgaria

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Bovine herpes virus 1 (BHV-1), known as causative agent of infectious bovine rhinotracheitis and infectious pustular vulvovaginitis, is widely spread in bovine population *(1)*. Clinical symptoms connected with respiratory, genital and central nervous system disorders have been reported *(2, 3, 4)*. The virus can infect the animals latently. Observations in Tanzania *(5)* have shown that BHV-1 is widespread among buffalo herds. Antibodies against BHV-1 (BHV-1 antibodies) were found in the buffalo sera with different percentage, namely 22% *(6)*, 63% *(7)* and 54.9% *(8)*. A high correlation (91.2%) was found between the indirect immunofluorescence and enzyme-linked immunosorbent assay (ELISA) results *(9)*. A passive haemagglutination test showed higher antibody titers (40.3%) in buffaloes with reproductive disorders in comparison to animals with respiratory disorders (29.2%). A higher sensitivity to BHV-1 was reported by the same authors in the Mura race and their crossbreeds than in the indigenous animals.

BHV-1 antibodies were identified in 58% of the buffalo sera investigated by a virus neutralization (VN) test *(10)*. Changes in dissemination and epizootology of BHV-1 after destruction of large animal farms in some Bulgarian regions were observed.

In this paper, we present comparative results obtained with buffalo sera from different Bulgarian regions by the VN test and ELISA.

A total of 457 buffalo sera from different farms situated in 5 different regions of the country were tested. The sera were heated at 58°C for 30 mins before testing.

The VN test was performed by the method described earlier *(11)* with some modifications. Aliquots (0.05 ml) of 2-fold dilutions of the sera in the Eagle's Minimum Essential Medium with Earle's salts were mixed with equal volumes of BHV-1 Ozet strain containing 100 $TCID_{50}$. After incubation at 37°C for 90 mins 4.10^4 calf trachea or MDBK cells were added. Negative and positive controls were included in each test.

Reagents for the ELISA were kindly donated by Prof. L. Ronsholt, National Institute for Virus Research, Lindholm, Denmark. Undiluted and serially 2-fold diluted sera were tested. Sera with both high and low titers of BHV-1 antibodies were included in each assay. The reaction was revealed with bovine biotinylated anti-IgG (BHV-1 IgG) avidin conjugated with peroxidase. Tetramethylbenzidin or orthophenylenediamine and hydrogen peroxide in 0.1 mol/l citrate buffer pH 5.0 was used as substrate. Then A_{450} and A_{620} (for reference) were read.

The ELISA results indicating the presence of BHV-1 antibodies represented ratios of the absorbance (A) values of the tested sera to those of the negative control sera expressed in %. A<50% was regarded as a positive, 50%<A<70% as a doubtful, and A>70% as a negative result.

*E-mail: lilly@iph.bio.bas.bg; fax: +3592-2542925.

Abbreviations: BHV-1 = bovine herpes virus; BHV-1 antibodies = antibodies against BHV-1; ELISA = enzyme-linked immunosorbent assay; VN = virus neutralization

Table 1. Percentage distribution of positively reacting animals by VN and ELISA tests in different Bulgarian regions

Test	Regions					
	Lch (n=18)	Rs (n=9)	Pd (n=54)	Tsh (n=70)	Sh (n=78)	Total (n=229)
VN (%)	22.2	33.3	31.5	24.2	19.2	26.8
ELISA (%)	28.1	36.9	34.5	29.1	24.3	30.6

In order to adjust for the normal wide variability of VN and ELISA titers of sera, relative changes of parameters from baseline studies were compared rather than absolute values.

Due to the small number of sera and the skewed distribution of results, the non-parametric two-tailed Mann-Whitney U test was used to determine the significance of differences in recalculated VN and ELISA titers, with $p < 0.05$ regarded as significant. The non-parametric Spearman rank correlation analysis of the VN and ELISA results was applied to 41 sera. In order to determine whether the ELISA had a higher sensitivity in comparison to the VN test, linear regression was performed between recalculated values of VN versus ELISA titers. The statistical analysis was carried out using the Stawin 5.1 software.

The highest serum dilution neutralizing virus growth in 50% of the wells was accepted as the neutralization titer. The optimum concentration of ELISA reagents (antigen, serum and conjugate) after using control positive and negative sera were determined by cross-titration.

The ELISA showed a higher percentage of positively reacting animals (30.6%) as compared to the VN test (26.8%). The sensitivity of the ELISA was significantly higher ($p < 0.001$) than that of VN test for 41 buffalo sera.

These results suggest circulation of BHV-1 among some buffalo herds in Bulgaria. The highest percentage of positively reacting animals in the ELISA and VN test was observed in two regions in Bulgaria (Table 1).

A high sensitivity ELISA was broadly used to detect antibodies against a large number of viruses. Using single-dilution quantitative assays for detection of BHV-1 some authors *(12, 13)* found a linear relationship between the results of ELISA and VN test. The elaboration and determination of specific conditions for each antigen-antibody system are usually ensured by a preliminary checkerboard titration of the ELISA reagents. Our results confirmed the higher sensitivity of ELISA in identification of BHV-1 antibodies in buffalo populations.

A herpesvirus isolated from the buffalo prepuce in Australia *(14)* was identified as BHV-1 by a VN test. The performed Southern blot analysis of virus genome *(15)* confirmed a close relationship between this herpesvirus isolate and BHV-1 at the protein level. This is the reason why a VN test could not distinguish these two viruses. The authors estimated approximately 85% homology in this case.

An earlier serological study conducted in 1988 in 8 regions in Bulgaria in large buffalo farms has shown 58% of positively reacting animals in VN test *(10)*. The lower percen-tage found by us in the present study (26.8%) was most probably due to destruction of large farms and dissemination of animals to small private farms. The decrease of direct and indirect contacts between viral carriers and susceptible receptive animals could be caused by the decrease of virus spread by the respiratory route.

The applied variant of ELISA indicated a large percentage of positively reacting animals and can be successfully used for serological diagnosis of BHV-1 among buffalo herds.

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