

ETIOLOGICAL AND MOLECULAR BIOLOGICAL INVESTIGATION OF CAPRINE HERPESVIRUS 1 ISOLATED IN BULGARIA¹

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Abstract

From goat and bucks with genital disorders and abortions the attempts for isolation of caprine herpesvirus 1 (CHV 1) were carried out. For virus exaltation Dexametazone was used. For viruses isolation vaginal, nasal, rectal, preputial swabs and organ samples were used. Primary and continuous cell cultures rabbit kidney (RK), Madin Darby bovine kidney (MDBK), and embrional bovine trachea (EBTR) were used for cultivation. For determination of DNA type and lipid envelop 60 µg/ml iod desoxyuridine (JDUR) and the ether treatment was used. Neutralization by specific hyperimmune serum obtained from Switzerland was performed. Five CHV 1 strains were isolated by cell cultures and identified as goat herpesviruses from different Bulgarian regions. After electron microscopy the viral agents with typical size and morphology for herpesviruses were established. For demonstration gC gene of CHV 1 the polymerase chain reaction (PCR) with primers designed from sequences deposited in gene bank were developed. Isolated on cell cultures herpesviruses were proved as caprine herpesvirus 1 by using applied PCR variant. The products after gC gene amplification from Bulgarian isolates were separated on the same place as the amplicons of reference CHV 1 strains.

Key words: CHV 1, PCR method, gC gen

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ETIOLOŠKO I MOLEKULARNO-BIOLOŠKO ISTRAŽIVANJE KOZIJEG HERPESVIRUSA 1 IZOLOVANO U BUGARSKOJ

Kratak sadržaj

Kod koza i jaraca sa genitalnim poremećajima i abortusima pokušali smo da izolujemo koziji herpesvirus 1 (CHV 1). Za reaktivaciju virusa korišćen je Dexametazon. Za izolaciju virusa korišćeni su vaginalni, nazalni, rektalni i prepucionalni bris. Za izolaciju – kultivaciju virusa su se koristile primarne i kontinuirane kulture ćelija bubrega zeca („rabbit kidney – RK”), bubrega goveda („Madin Darby bovine kidney – MDBK”) i embrionalne traheje goveda („embrional bovine trachea – EBTR”). Za određivanje DNK tipa i lipidnog omotača upotrebljeno je 60 µg/ml jod dezoksiuridina (JDUR) i tretiranje etrom. Urađena je neutralizacija specifičnim hiperimunim serumom koji je dobijen iz Švajcarske. Pet CHV 1 sojeva iz različitih oblasti Bugarske je izolovano na kulturama ćelija i identifikovano kao koziji herpesvirus. Nakon elektronske mikroskopije virusni agensi tipične veličine i morfologije su prepoznati kao herpesvirus. Za dokazivanje gC gena CHV 1 upotrebljena je lančana reakcije polimeraze (PCR) sa prajmerima koji su dizajnirani na osnovu virusnih sekvenci prijavljenih u banci gena. Upotrebom primenjene PCR tehnike, na kulturi ćelija izolovani herpesvirusi su potvrđeni kao koziji herpesvirus 1. Sekvence umnoženih gC delova genoma bugarskih izolata su se locirale na istom mestu u filogenetskom stablu kao i sekvence referentnih CHV 1 sojeva.

Ključne reči: CHV 1, PCR metod, gC gen

INTRODUCTION

Caprine herpesvirus 1 (CHV 1) is a DNA containing virus with a diameter 120-150 nm and lipid envelope. The antigenic peculiarity of virus is not well studied. According to Engels et al. (1987) viral DNA has a high homology with bovine herpesvirus 1 DNA. After investigation of agent by neutralization test, ELISA and restrictase fragment pattern analysis is determined that CHV 1 is immunologically different from herpesviruses in big ruminants and elks (5, 10, 18). Glycoproteins with molecular weight 74 and 91kD are responsible for cross neutralization between bovine and caprine herpesvirus. Despite the differences in molecular weight it was de-

terminated by PAGE that the polypeptides of goat and bovine herpesviruses have similar mobility (2).

The first report for CHV1 comes in early 1974 from Saito et al. (13) in California who described the disease with high percent mortality among angora goats. In young animals CHV 1 caused generalized infection damaging gastrointestinal and respiratory tracts (1). Macroscopically are visible ulcer and necroses in all gastrointestinal channels, changes in lungs, urinary bladder and liver. Pathohistologically are observed heavy necrotizing enteritis, as well as thickening of alveolar septa and necrotic bronchoalveolitis (12). Clear microscopic damages are observed also in liver, urinary bladder, spleen, thymus, mesenterial lymph nodes and kidney. The disease is accompanied with diarrhea, rhinitis, tracheitis, breathing disturbance and later suppurative nasal discharges.

In bucks the virus caused balanopostitis and penopostitis and in goats vulvovaginitis (8, 14, 15). From damaged genital tract the infection is transmitted by breeding. Abortions are observed, too (17, 19).

The number of isolated CHV 1 in the world is limited. Some parts of epidemiology, pathogenesis and spreading of the disease are not well elucidating. This is the reason to isolate etiological agent and study the cultural and genome characteristics.

In this paper is described the isolation and identification of CHV 1 causing goat infection and by using the physicochemical and molecular biological methods to study some biological peculiarity of the virus.

MATERIAL AND METHODS

Nasal, vaginal and preputial swabs, lung lavages, 10% suspension in phosphate buffered saline (PBS) from internal organs, probang test, buffy coats, fecal and milk samples were used for virus isolation. Totally 163 samples were checked. The samples originated from several country regions: Troian, Suhindol, Kustendil, P.Bania, Smolian, Haskovo, Yambol.

For herpesvirus isolation the method for exaltation by dexamethasone (DMSO) was used (3).

Cell cultures, media and solutions

For virus isolation and identification of viruses primary and permanent cell cultures were used. The primary cell cultures were obtained newborn rabbit kidney and as permanent cell lines Madin Darby bovine kidney (MDBK), (AUBEK), Georgia bovine kidney (GBK), embryonic bovine trachea (EBTR) and calf trachea (TTr). For washing cell culture PBS with rN 7.4 or normal saline were used. As growing media Eagle's Minimum Essential Medium (MEM) in Hanks balanced salts solution supplemented with 10% fetal calf sera (FCS) and as a supportive media the same media with 2% FCS were used. As additives penicillin 100 UI/mL, streptomycin 100 /mL, 0.2 M/L L – glutamine and NaHCO₃ were added.

Isolation and identification of CHV 1 on cell cultures

For infection cell cultures with complete monolayer were used. After elimination of media and washing with PBS or normal saline 0.2 mL virus inoculums were added. Depending on the type of samples adsorption varied between 60 and 120 min at temperature of 37°C. After washing of cell monolayers and adding the maintaining media the tubes were placed in a roller at 37 °C. Simultaneously, with the investigated samples non infected control cells were used in the same condition.

Microscopically, the isolates growth was controlled daily by determination the presence of cytopathic effect (CPE) on monolayers. In attempts for primary isolation the cell cultures were observed microscopically 7 days after the infection. Three consecutive passages were performed when CPE was missing and more passages - when CPE was visible. Because of the difficulties connected to the primary isolation of CHV 1 other methods for cultivation were used – infection on fresh monolayer, young 24 h cell cultures or in cell suspension.

Identification of viral agents in the infected cell monolayers with CPE were accomplished by the methods described by Payment and Trudel (1993). To determine the nucleic acid type the viral isolates were treated with 60 µg/mL 5-jodo-2-deoxyuridine (JDUR), and for lipid presence treated with 20% ether.

In neutralization reaction -variant the reference hyperimmune sera from Switzerland had titer of 8 log₂. The observed CPE was similar to CHV. The serum participated in maintain media at constant dilution 1:4, and investigated isolates in 10 times increasing dilution. As control the reference CHV 1 strain E/CH with a titer 10^{7.33} TCID₅₀/mL on MDBK was applied. As control heterologous RNA strain paramyxovirus parainfluenzae 3 virus "Svetovrachene" strain was used.

Electron microscopy

Electron microscopy investigation was performed with direct electron microscopy (DEM) after slow speed centrifugation of viral isolates 2000 rpm/20 min. Supernatants were differentially centrifuged for 1h at 30000xg and the obtained pellet were dissolved in minimal volume sterile distilled water – 0.5 ml, after which new slow speed centrifugation was carried out at 2000 rpm/20 min. For performance of DEM the clear supernatants were added onto butvar and carbon coated copper grids with 400 mesh and negative staining were performed with 2% sodium phosphotungstate rN 5.8 or 2% uranyl acetate rN 4.2-4.5.

The investigations were carried out by electron microscope JEM 1200 EX with accelerated tension 80 kV and instrumental enlargement 40000 to 75000X.

Molecular biological investigation

The viruses were cultivated in cell culture and in 80% visible CPE they were harvested. Commercial kit GIAamp DNA mini kit Giagen, GmbH, Hilden Germany was used for isolation of DNA following the kit instruction. The DNA amount was con-

trolled spectrophotometrically with Jenway apparatus (Genova) and by gel electrophoresis and was amplified by polymerase chain reaction (PCR) using the following pairs of primers corresponding to sequences 759–779 and 1172–1154 from the gene bank:

Forward R₁ 5' - AGGGCGCCGGTGGATGCTCTG - 3'

Reverse R₂ 5' - GGCGGGCGGTGCGTCGTGA - 3'

Reaction was conducted in a volume of 25 µL, using Fidelity tag PCR Master mix (2X), forward primer P₁, reverse primer P₂ and DNA template. Thermocycler QB - 96 (LKB) and the program described from Hecht et al. (1995) were used for PCR reactions.

Heated lid 110°C

1 cycle

Denaturation 96°C-10 min; Annealing 65°C-1 min; Elongation 72°C-1 min.

Following from 35 cycles

Denaturation 95°C-1 min; Annealing 65°C-1.30 min; Elongation 72°C-1 min.

Final Elongation at 72°C for 5 min.

DNA extraction and PCR products were electrophoresed on 2% agarosa with ethidium bromide (1mg/ml) and were visualized by UV transilluminator. For PCR specificity closely related IBR "Ozet" strain were included.

Serological investigation by micro virus neutralization test (MVNT)

Sera from goats with clinical symptoms typical for CHV 1 infection and recovered were investigated for antibodies against CHV 1 by micro virus neutralization test (MVNT) - variant. The sera were temperature treated at 56°C for 30 min, after that serial two fold dilution in maintenance media MEM Eagle, penicillin 100 UI/mL, streptomycin 100 µg/mL, 2 mM/L L-glutamin, 1.5 g/L sodium bicarbonate were performed. Hundred tissue culture infectious dose 50/mL (TCID₅₀/mL) from reference strain E/CH with a titer 10^{7.3} was added to dilute sera. The mixtures were incubated at 37°C for 2 h. After that the indication system - cell line MDBK at quantity 4h10⁻⁴ cells/mL was added.

The account of results was performed till 72 h. The highest dilutions of sera giving complete growth suppression of indicator virus were accepted as serum titer.

RESULTS

Viral agents were isolated only after dexametazone treatment from animals with antibodies against CHV 1. The viruses were isolated only from vaginal and preputial swabs. The virus isolates have cultural and biochemical characteristics typical for caprine herpesvirus 1. From goats and bucks with problems in breeding 5 CHV 1 strains were isolated: Troian, Suhindol, Kustendil, P. Bania and Biser.

The citopatic effect on primary and permanent cell cultures depended on used cell lines and started with rounding of cells. Between 6 and 12 h after infection of monolayers only single cells were rounded. Cytopatic effect on 24 h became more diffuse and great number of cells was damaged (Fig. 1A) after which the speed changes

on monolayers were visible. At 48 h on cell monolayers were observed extended empty places as a result of the viral growth (Fig. 1B) and after 48 h the part of cell monolayers was detached from the tube walls and after 72 h a destruction of monolayers was visible (Fig.1 C). In control non infected MDBK cell cultures the cytopathic effect was not observed (Fig. 1 D)

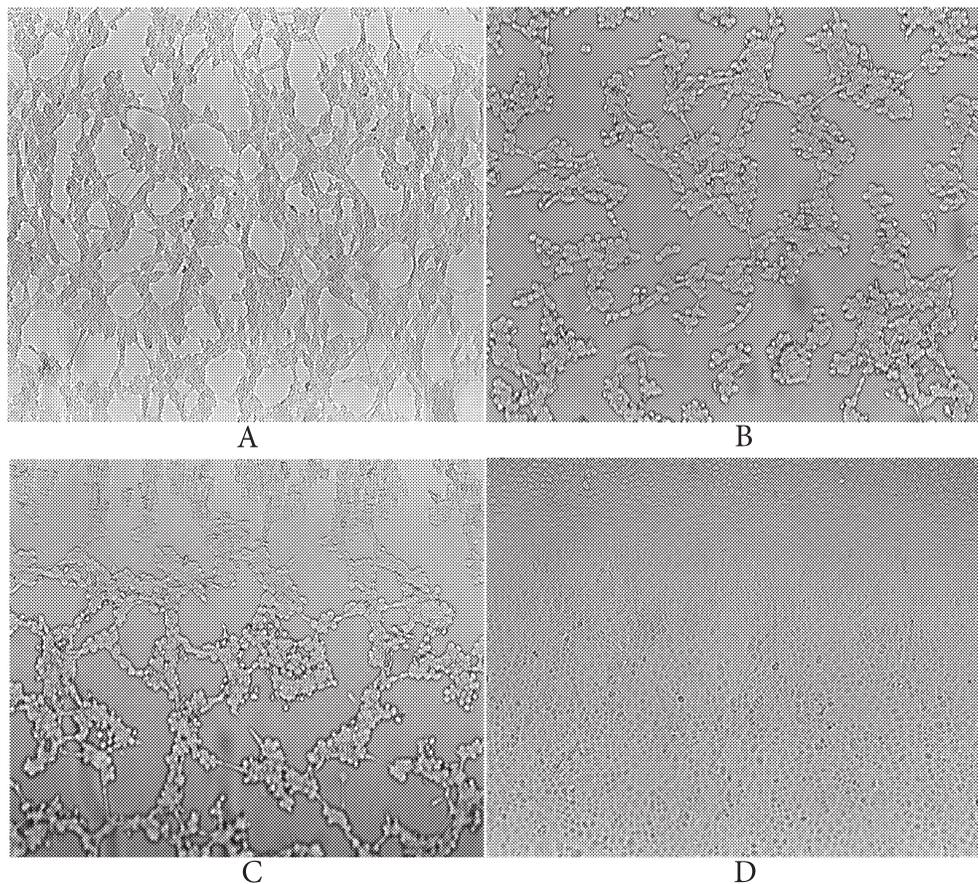


Fig. 1. Cytopathic effect of isolate "Suhindol" on cell culture MDBK: A - 24 h after infection, B - 48 h post infection, C - 72 h after infection, D - control non infected cell culture MDBK. Magnification - 200H.

The obtained results during investigation for identification of agents in infected cell cultures are shown in Table 1.

Table 1. Data from biochemical and molecular biological studies for determination of nucleic acid type, effect of HIS against CHV 1, presence of lipid coat, impact of low and high pH buffers with five CHV 1 isolates, reference E/CH strain and heterologous Pi-3 strain "Svetovrachene".

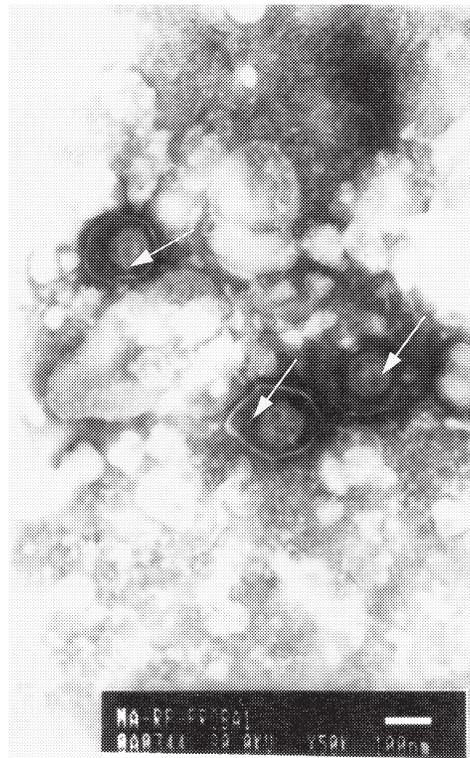
Sample	Strain titration after treatment with				
	Titers before treatment	60 ml JDUR	HIS anti CHV 1	20% ether	Buffer with rN<3 and > 8
goat "Trojan"	$10^{-7.32}$	$10^{-3.0}$	10^{-1}	-	-
7892 goat "Suhindol"	$10^{-6.33}$	$10^{-2.33}$	$10^{-1.50}$	-	-
buck "Kustendil"	$10^{-5.66}$	$10^{-2.0}$	-	-	-
buck "P. Bania"	$10^{-6.33}$	$10^{-3.0}$	-	-	-
buck "Biser"	$10^{-4.66}$	$10^{-2.0}$	-	-	-
Referent strain E/SN	$10^{-7.33}$	$10^{-2.33}$	$10^{-1.33}$	-	-
Pi-3 "Svetovrachene"	$10^{-5.33}$	$10^{-5.33}$	$10^{-5.33}$	-	-

After treatment with 60 /mL 5-jodo-2-deoxyuridine (JDUR) for the determination of nucleic acid type in 5 isolates a suppression of growth with more than 3-4log₁₀ was established. In treatment of referent strain E/SN a suppression of growth - 5 log₁₀ in comparison with non treated virus. Suppression of viral titer was not found in RNA strain Paramyxovirus PI-3 "Svetovrachene". After neutralization test with positive hyperimmune sera from Switzerland in all new isolated strains a suppression between 4-6 log₁₀ was obtained. In reference strain E/CH also was determined reduction in viral titer with 6 log₁₀. In heterologous strain "Svetovrachene" such decrease in viral titer was not observed.

The five isolates, referent E/CH and control RNA viral strain were not growing on cell culture after treatment with 20% ether.

After electron microscopy the viral particles with diameter approximately 150-180 nm and typical for herpesviruses morphology were observed (Fig. 2).

Fig. 2. Electron microscopy of viral isolate "Suhindol". With arrows are noted viral particles.



In molecular biological investigation the quantity of DNA obtained by Giagen columns varied between 163.5 ng/ μ l and 198.2 ng/ μ l. After using PCR master mix with the described program multiplication of gC gene was possible. Products with size 414 bp for all 5 caprine herpesviruses isolated in Bulgaria and for all reference strain from Europe and USA were obtained after performing PCR reaction (Fig. 3). There was not multiplication of genome of investigated IBR "Ozet" strain in using the same primers and procedure for PCR specificity.

Fig. 3.



Fig. 3. Polymerase chain reaction of 5 Bulgarian isolates and reference CHV 1 strains. Lane M, 1-kb ladder as a size marker, lane 1-"Troyan", lane 2-"Suhindol", lane 3-"P. Bania", lane 4-"Kustendil", lane 5-"E/CH", lane 6-"McKercher", lane 7-"Sp-1", lane 8-"Sp-2", lane 9-"Biser", lane 10-"IBR-Ozet", lane 11-non infected cell culture -MDBK.

After serological investigation in five country regions from which the viruses were isolated an increase of antibody titers two and more \log_2 in paired sera was determined against referent E/CH strain (Fig. 4).

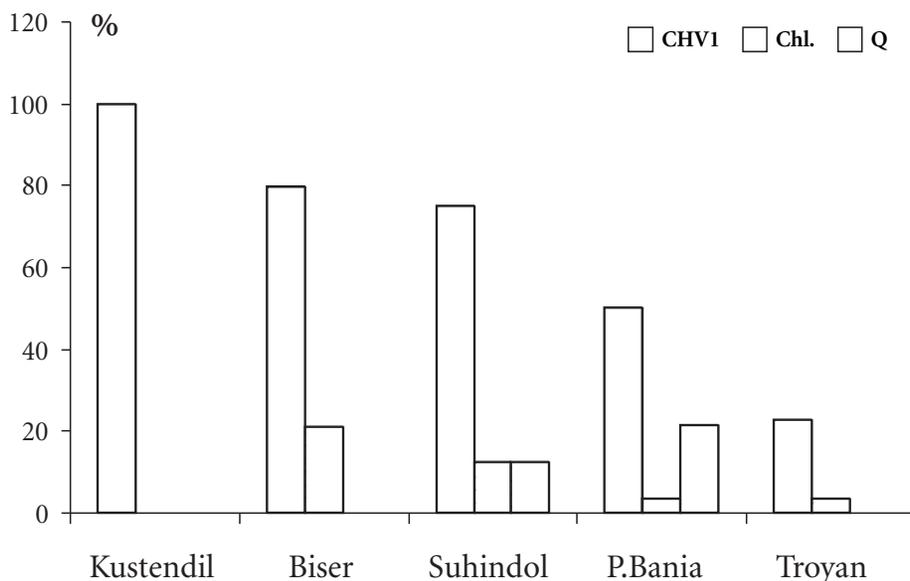


Fig. 4. Percent distribution of positive serum samples against referent E/CH strains and Chlamydomphilla and Q-fever in investigated flocks.

The highest percent positive sample in Kustendil (100%) followed by Biser (79.9%), Suhindol (75%), P. Bania (50%), and Troian (22.75%) were found (Fig. 4). The number of positive bucks increased after breeding campaign, and also the titer of antibodies was increased in comparison with antibody titers before the campaign. In some goats were determined repeating twice and three time heat especially in Troyan, Suhindol and P. Bania farms. Additionally titers against chlamidophilla pursue infection were determined for Troyan (3.44%), Suhindol (12.5%), P. Bania (3.57%), Biser (21%) and against coxiella burnety in Suhindol (12.5%) and P. Bania (21.42%) only.

DISCUSSION

Using the scheme (Buonavoglia et al., 1996) for exaltation of caprine herpesvirus 1 we succeed to isolate five CHV 1 strains from vaginal and preputial swabs. The virus isolated only from vaginal swabs, but not from nasal, rectal swabs and buffy coat. The isolation of goat and bucks viruses was possible with high DMSO doses between 3 and 9 days after finishing the treatment of positive for antibodies animals. Smaller DMSO doses were not enough for virus isolation.

The cytopatogenic effect in the adaptation and cultivation of strains was with mild changes with rounding of the cells after that the monolayer was destructed as described by Engels et al. (1983) changes. The grow rate of new isolated CHV 1 strains by us was more rapid in comparison with that of bovine herpesvirus 1 as Engels et al. (1983) established for CHV 1 strain. After latent period of 5 h exponential phases of virus growing 6 to 12 h after infection was observed. The cytopatic effect of isolated viral agents on cell cultures with rabbit and bovine origin was more diffuse than the

bovine herpesviruses. Most probably that is the results of higher citolitic properties of CHV 1 in comparison with BHV 1.

After treatment with JDUR and the applying of MVNT with hyperimmune sera from Switzerland against reference CHV 1 strain the viral titers were decreased with more than 2-4log₂. This is the evidence that the isolated viral agents were CHV 1. The complete suppression of viral growth after treatment with ether confirmed that the new isolated samples and reference controls are with lipids bilayers of membrane. This is supported by the fact that the heterologous Pi 3 virus is suppressed from a treatment with 20% ether. At lower and higher pH solutions the growth of viral isolates is also suppressed. The probable reason for this is the repression of contacts between viral antigens and cell receptors and the impossibility for entrance and multiplication of causative agents in cell cultures.

At electron microscopy studies viral agents with typical size and morphology for herpesviruses were determined.

The observed biological peculiarities of all 5 Bulgarian isolates by performed PCR reaction similar to reference CHV 1 strains E/CH and McKercher are the evidence that all five isolates are CHV 1 strain. This permit us to conclude that PCR reaction with the described primers and procedures can be used for rapid and exact diagnosis.

After investigation by MVNT of sera originating from animals in acute and convalescent period of the disease from the five farms we detected an increase in antibody titers with two and more logarithms against reference CHV 1 strain which is an indication for CHV 1 circulation in flocks. On some farms the established antibody titers were a result of single infection, while on other farms it was accompanied with chlamydomydia and q-fever infections. The disease elapsed more heavily in the presence of these accompanying infections and recovering period of animals was long lasting and often in next breeding season the breedings were hampered.

CONCLUSIONS

1. Isolation and adaptation of five goat herpesviruses were possible only after treatment with DMSO.
2. CHV 1 viral strains can be successfully adapted on primary and permanent cell culture of bovine origin.
3. Isolated viral agents have cultural, biochemical and morphological characteristics typical for CHV 1.
4. The amplicons of all virus strain after performed PCR assay have the same size and mobility as the reference CHV 1 strains.
5. Statistically significant increasing of MVNT antibody titers in convalescent sera is the evidence for circulation of CHV 1 on goat farms.

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