Elsevier Editorial System(tm) for
Comparative Immunology, Microbiology & Infectious Diseases
Manuscript Draft

Manuscript Number:

Title: Goats are susceptible to Bubaline herpesvirus 1 infection: results of an experimental study

Article Type: Full Length Article

Keywords: Bubaline herpesvirus 1; Epidemiology; Experimental infection; Goats; Susceptibility; PCR.

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Abstract: Herpesvirus infections are generally subjected to strong host species restriction, although virological and serological investigations have revealed the possibility of cross-species infections in closely related animal species. In this study we evaluated susceptibility of goats to infection by Bubaline herpesvirus 1 (BuHV-1). Four goats were inoculated intra-nasally with BuHV-1 and monitored clinically, virologically and serologically for 42 days. None of the goats displayed clinical signs althoughall the animals variably shed the virus by the nasal route during the first 12 days after infection. BuHV-1 was also detected in the white blood cells of two animalsin the first week post infection. The results suggest that goats are susceptible to BuHV-1 infection and that they could play an epidemiological role in the circulation/transmission of the virus among domestic and wild ruminants and impact to some extent on the control plans for herpesviruses in cattle.

UNIVERSITÀ DEGLI STUDI DI BARI ALDO MORO

DIPARTIMENTO DI MEDICINA VETERINARIA

To The Editor in chief
Comparative Immunology,
Microbiology & Infectious Diseases
Prof. Henri-Jean Boulouis

Dear Editor,

I submit to your judgement the manuscript "Goats are susceptible to Bubaline herpesvirus 1 infection: results of an experimental study" by Camero M., Larocca V., Losurdo M., Lorusso E., Patruno G., Staffa V.N., Martella V., Buonavoglia C., Tempesta M., for publication on Comparative Immunology, Microbiology & Infectious Diseases.

As the corresponding author and on behalf of the other authors, I declare that the manuscript is original and has not been simultaneously submitted for publication in another journal.

Yours sincerely,

Maria Tempesta

Valenzano 19th September 2016

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Highlights

- Susceptibility of goats to Bubaline herpesvirus 1 infection
- Lack of clinical signs in goats after Bubaline herpesvirus 1 infection
- Epidemiological importance and impact on herpesvirus circulation
- Inclusion of goats in the control program

Original article

Goats are susceptible to Bubaline herpesvirus 1 infection: results of an experimental study

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Abstract

Herpesvirus infections are generally subjected to strong host species restriction, although virological and serological investigations have revealed the possibility of cross-species infections in closely related animal species. In this study we evaluated susceptibility of goats to infection by Bubaline herpesvirus 1 (BuHV-1). Four goats were inoculated intra-nasally with BuHV-1 and monitored clinically, virologically and serologically for 42 days. None of the goats displayed clinical signs although all the animals variably shed the virus by the nasal route during the first 12 days after infection. BuHV-1 was also detected in the white blood cells of two animals in the first week post infection. The results suggest that goats are susceptible to BuHV-1 infection and that they could play an epidemiological role in the circulation/transmission of the virus among domestic and wild ruminants and impact to some extent on the control plans for herpesviruses in cattle.

Key words: Bubaline herpesvirus 1, Epidemiology, Experimental infection, Goats, Susceptibility, PCR

1. Introduction

Bubaline herpesvirus 1 (BuHV-1) is an alpha-herpesvirus isolated in 1971 in Australia [1]. The virus was subsequently identified in Italy [2] and, more recently, in Argentina [3], indicating a worldwide distribution.

BuHV-1 induces subclinical disease in water buffalo (*Bubalus bubalis*), which, thus far, is regarded to as the primary virus host and reservoir. Recently, the DNA of BuHV-1 was detected by PCR in the tissues of an aborted fetus of a water buffalo suggesting a possible association of the virus with abortion [4].

Upon genome sequencing, BuHV-1has been found to be highly related to Bovine herpesvirus 5 (BoHV-5), responsible for meningo-encephalitis in calves and, to a lesser extent, to Bovine herpesvirus 1 (BoHV-1), which is associated with infectious bovine rhinotracheitis (IBR) and infectious pustolar vulvovaginitis (IPV) [5]. BoHV-1/BoHV-5 and BuHV-1 display a sequence homology ranging from 87-93 % [6] and 97% [4].

In nature, most herpesviruses are strictly associated with a specific host species, and almost all the animalspeciessuffer from infections by at least one herpesvirus species. This situation suggests that the herpesviruses have mainly co-evolved with their hosts, leading to a close adaptation [7]. Cross-species infection studies with different ruminant alphaherpeviruses have been performed in cattle and serological investigations have showed a correlation among ruminant alphaherpeviruses. In particular, the potential role of ruminant species other than cattleas BoHV-1 reservoir has been investigated thoroughly [8], [9], [10], [11], [12], [13] and [14]. Furthermore, previous experimental studies that BoHV-1 vaccine-induced antibodies are able to cross-protect goats and buffaloes against caprine herpesvirus 1 (CpHV-1) [15] and BuHV-1 [4].

However, the literature on susceptibility of ruminants other than buffaloes to BuHV-1 infection is limited and there is no information at all on susceptibility of small ruminants to this

virus. This could have important implications for BuHV-1 epidemiology and ecology. In order to evaluate whether goats are susceptible to infection by BuHV-1, in the present study we experimentally infected goats with a BuHV-1 isolate.

2. Materials and methods

2.1.Experimental Design

Four adult goats, two females (A, B) and two males (C, D), seronegative to caprine CpHV-1, BoHV-1 and BuHV-1, belonging to flocks brucellosis and CpHv-1 infection free, were used.

The experiment has been carried out in the Isolation Unit of the Department of Veterinary Medicine of the University of Bari according to the National Guide for Care and Use of Experimental Animals. The experiment has been approved and authorized by the Body responsible for animal welfare (OPBA) of the University of Bari and Ministry of Health (aut. n. 852/2015).

A Bubaline herpesvirus 1 (BuHV.1) strain gently supplied by prof. E. Thiry (University of Liege, Belgium), grown on Madin Darby bovine kidney cells (MDBK) developed in Dulbecco-Minimal Essential Medium (D-MEM) with 10% of bovine fetal serum was used. The titer of stock virus was $10^{6.75}$Tissue Colture Infectious Dose $(TCID)_{50}$/50µl.

All goats were infected by nasal route with 3mls (1,5 mls/nostril) of undiluted stock virus of BuHV-1 and housed in separate boxes. During an observation period of 42 days the goats were examined daily for general and local clinical signs and body temperature was recorded for 14 days. Starting the day before the infection and then for 20 days post infection (d.p.i.), nasal, ocular, rectal and genital swabs were collected for the virological investigations (isolation on MDBK cells and PCR assay); heparinized blood samples for buffy coat were also daily collected to detect viral DNA by PCR assay. Blood samples were taken the day before the infection and then at 0, 7, 14, 21, 28 and 42 d.p.i.to evaluate the antibody response to BuHV-1 by a neutralization assay.

2.2.Virus isolation and titration

Swabs were dipped in 1.5 ml of D-MEM and centrifuged at 5,000 × *g* for 5 min. The supernatant was treated with a 10% mixture of antibiotics (5,000 IU/ml penicillin, 2,500 μg/ml streptomycin, and 10 μg/ml amphotericin B) for 30 min at room temperature, diluted in serial 10-fold steps, and inoculated in quadruplicate onto MDBK cells in 96-well microtiter plates. The plates were read after 3 days of incubation, and the viral titers were calculated by Reed and Muench method [16].

White blood cells were isolated from heparinized blood samples using the standard density-gradient separation procedure (Lympholyte, CEDARLANE laboratories Ltd., Burlington, NC, USA) and washed twice with RPMI medium before the use.

2.3.DNA extraction and PCR

Viral DNA was extracted from swabs and buffy coats using the commercial QIAamp tissue kit (Qiagen GmbH, Hilden, Germany), according to the instructions of the company. The PCR was carried out using a pair of primers (BuHV1F 5'- GGCGGTGCAGGTGTAGTC- 3'; BuHV1R5'-CTCGCGCACGTCCGTCCTCACGCT- 3') constructed on a sequence of 360 bp of the gene coding for the glycoprotein C (gene UL44) of BuHV-1 (accession nr. KF679678) (unpublished data). The PCR was carried out in a total volume of 50μl containing 5 μl of DNA sample, 2 μl of 10× PCR buffer, 1.5 mM $MgCl_2$, 1.25 mM of each oligonucleotide triphosphate, 200 μM of each primer, 1.5 U of Takara LA Taq (Takara Bio, Inc.), and 5 μl of DMSO and sterile water up to 50μl. The thermal profile consisted of 94°C at 2 min and 40 cycles at 94°C for 1 min (denaturation), 55°C for 1 min (annealing), and 68°C for 1 min (polymerization), followed by a final extension at 68°C for 10 min. Ten microliters of the PCR products was analyzed by electrophoresis in 1.5% agarose gel after staining with GelRed™ (Biotium, Hayward, CA, USA) and visualized by UV trans-illuminator equipped with software data acquisition and image processing (Gel Doc, Biorad, Segrate Milan, Italy).

The specificity of the PCR assay was evaluated on BoHV-1 and CpHV-1 strains obtaining negative results. Serial 10-fold dilutions of BuHV-1 viral suspension were tested to evaluate the sensitivity of the PCR and viral DNA was detected in 10^{-6} dilution of the stock virus.

2.4. Serological analysis

Serial twofold dilutions of serum samples from 1:2 to 1:256, were mixed with 100 $TICD_{50}$ of BuHV-1 in 96-well microtitre plates. The plates were held for 90 min at room temperature and 20,000 MDBK cells were then added to each well. Reading was done after three days of incubation at 37°C in presence of 5% CO_2. The titre of each serum was expressed as the highest serum dilution neutralizing the virus.

3. Results

3.1.Clinical examination

None of the goats showed general or local clinical signs. Two animals (B and C) had a slight transient arise of temperature (39.6°C) on d.p.i. 8 (goat B) and on d.p.i. 3 and 4 (goat C).

3.2.Virological results

The virological results are reported in the fig. 1.

Goat A shed virus only from nasal swabs from the 4^{th} to the 8^{th} d.p.i., with the peak of viral excretion ($10^{3.50}$ $TCID_{50}$/50 µl) at the $6t^{h}$d .p.i. Viral DNA was detected by PCR from nasal swabs from the 1^{st} up to the 10^{th} d.p.i. All ocular, rectal and vaginal swab resulted constantly negative for virus isolation and DNA detection by PCR assay. Viral DNA has been detected in the buffy coats from the 1^{st} to the 7^{th} d.p.i.

Goat B shed virus only from nasal swabs from the 2^{nd} to the 10^{th} d.p.i., with the peak of viral excretion ($10^{3.50}$ $TCID_{50}$/50 µl) at the5^{th} d.p.i.. Viral DNA was detected by PCR from nasal swabs from 1^{st} to 11^{th} d.p.i. All ocular, rectal and vaginal swabs resulted constantly negative for virus

isolation and DNA detection by PCR assay. Viral DNA has been detected in the buffy coats from the 1st to the 7th d.p.i.

Goat C shed virus only from nasal swabs from the 1st to the 8thd.p.i., with the peak of viral excretion ($10^{5.00}$ $TCID_{50}$/50 µl) at the 2ndd.p.i.Viral DNA was detected by PCR from nasal swabs up to 11thd.p.i. All ocular, rectal and prepucial swabs resulted constantly negative for virus isolation and DNA detection by PCR assay. BuHV-1 DNA was not detected in the buffy coat.

GoatD shed virus only from nasal swabs from the 2nd to the 12th d.p.i., with the peak of viral excretion ($10^{5.50}$ $TCID_{50}$/50 µl) at the 4th d.p.i. Viral DNA was detected by PCR from nasal swabs up to13th d.p.i. All ocular, rectal and prepucial swabs resulted constantly negative for virus isolation and DNA detection by PCR assay. BuHV-1 DNA was not detected in the buffy coat.

3.3.Neutralization test

All the goats were negative for antibodies to BuHV-1 at d.p.i. 7. At d.p.i. 14, goats C and D showed an antibody titer of 1:2, goat A1.4 and goat B 1:8. At d.p.i. 21 the antibody titers arose to 1:4 in goats C and D whereas in goat A and B remained unchanged. At d.p.i. 42, the titers resulted at the same levels detected at d.p.i. 21 (table 1).

4. Discussion

Usually, heterologous viral infections (i.e. infections of a virus common in a given animal host in another host species) result is asymptomatic to mild-symptomatic infections, due to the existence of host species barriers that restrict virus replication and naturally attenuate virus pathogenicity [17] and [10]. In some cases, however, heterologous infections may determine severe clinical pictures in the new host. Cercopithecine herpesvirus 1, also known as B virus or McHV1 [18] is an alpha-herpesvirus indigenous in Asiatic macaques. In its natural host, McHV1 infection strictly resembles infection of Herpesvirus simplex 1 and 2 in humans. However, transmission of McHV1 to non-macaque primates, including humans, can result in serious and often fatal

188 encephalomyelitis [19]. Accordingly, as predicting the behavior of heterologous herpesvirus
189 infections is not possible, *in vivo* experiments are required to assess more precisely what happens
190 when heterologous herpesvirus infections occur.

191 The data generated in the present study suggest that goats are susceptible to BuHV-1
192 infection even if the infected animals did not develop any clinical signs. Indeed, after nasal
193 administration, the virus was shed nasally for several days with relatively high titers. Although a
194 few animals were used in the experiments, all the animals shed the virus only by the nasal route.
195 Although viral DNA was detected during the first week post infection in the buffy coats of two of
196 the four infected animals (goats A and B), systemic spread and dissemination of the virus was not
197 observed since BuHV-1 was not shed by other routes (ocular, genital, rectal) than the nasal one.
198 Starting from the 14^{th} d.p.i., neutralizing antibodies were found in the sera of all the animals,
199 indicating active sero-conversion.

200 Susceptibility of goats to BuHV-1 infection may be of relevance under an epidemiological
201 point of view. A major concern derived from interspecies circulation of herpesviruses in ruminants
202 is related to the increased interest in IBR eradication programs in cattle in Europe due to the
203 significant losses in bovine livestock industry. Eradication programs must necessarily take into
204 account the possibility of infection of cattle by heterologous, yet closely related, herpesviruses from
205 other ruminants. Also, eradication plans should consider the possibility of contact of cows with
206 heterologous hosts harbouring BoHV-1. For instance, BuHV-1, BoHV-5, CpHV-1, rangiferine
207 herpesvirus 1 (RanHV-1) and cervine herpesvirus 1 (CerHV-1) are antigenically and genetically
208 strictly related to BoHV-1 and they are all able to cross the host species barrier. This may challenge
209 the serological diagnostics because of the lack of specific assays able to discriminate between
210 BoHV-1 and closely related herpesviruses from other ruminants. Although improvements have been
211 reached using ag B/gE Elisa assay [20] and [21], thus far serological tests able to discriminate
212 between BoHV-1 and BuHV-1 are not available [14]. The presence of BoHV-1 related
213 herpesviruses in ruminants reared in the same areas where IBR is under control could impose

unjustified restriction measures in ruminant trading and, at the same time, the presence of heterologous species bearing BoHV-1 may hinder eradication of IBR infection. Indeed, in extensive farms more ruminant species may enter in contact, thus enabling the inter-species circulation of pathogens. Several pieces of evidence indicate that transmission of herpesviruses among ruminants is not uncommon [22].

5. Conclusions

In this scenario, and in the view of the results of our experiments, the possibility of natural transmission of BuHV-1 from buffaloes to goats and vice versa can be hypothesized. Whether goats may act as a reservoir for BuHV-1, as already demonstrated for BoHV-1 [23], [8] and [10], should be considered. In addition, other pathogenetic pathways of BuHV-1 in goats should be investigated with attention, including: i) the ability of BuHV-1 to cause reproductive disorders when the infection overlaps with gestation and ii) the ability to trigger latent infections and to reactivate under certain conditions.

Altogether, these pieces of information will be useful to understand more in-depth the ecology of BuHV-1in domestic and wild ruminants. Further investigations would be carried out to assess the intra- and interspecies transmission of the virus. Also they will be useful, in the future, when devising prophylaxis plans for the control of herpesvirus infections in buffaloes.

Acknowledgements

The Authors would like to thank Dr. Carlo Armenise for his expert technical assistance.

Funding: This work was supported by the funds of the University of Bari "Fondi Ateneo ex 60% 2014"

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Figure Legends:

Fig1. Viral titers on MDBK cells from nasal swabs of goats intranasally infected with BuHV-1

313

314

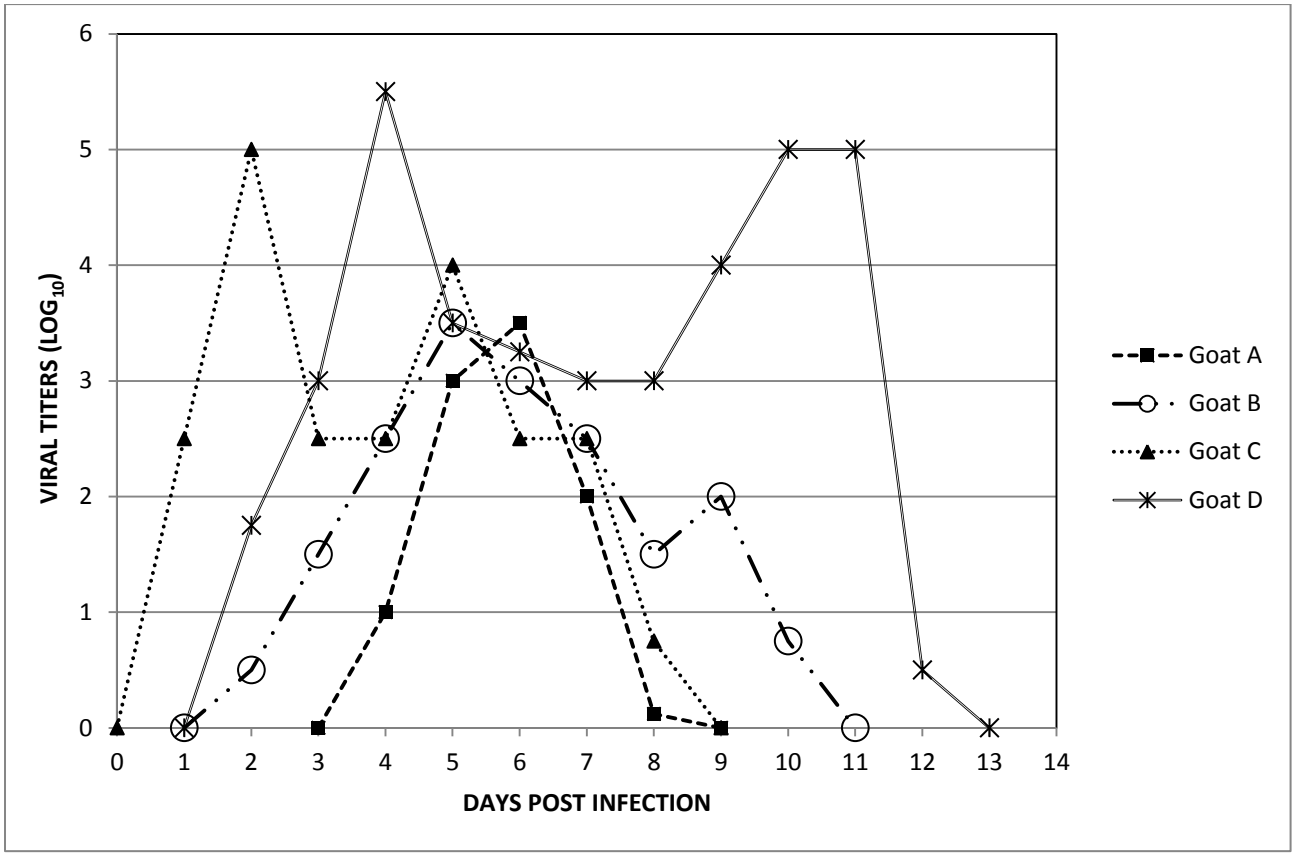
Table

Table 1. Neutralizing antibody titers in goats after intranasal infection with BuHV-1.

GOAT	0*	7	14	21	28	42
A	<2	<2	1:4	1:4	1:4	1:4
B	<2	<2	1:8	1:8	1:8	1:8
C	<2	<2	1:2	1:4	1:4	1:4
D	<2	<2	1:2	1:4	1:4	1:4

* days post infection

Figure


VIRAL TITERS (LOG10)
DAYS POST INFECTION
Goat A
Goat B
Goat C
Goat D
0
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