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Detection of neutralizing antibodies against bluetongue virus serotype 8 by an optimized plasma neutralization test

Gabriella Worwa*,[1], Valérie Chaignat, Julia Feldmann, Barbara Thür

Institute of Virology and Immunoprophylaxis, Sensemattstrasse 293, CH-3147 Mittelhäusern, Switzerland

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The neutralization test is used commonly for quantifying neutralizing antibodies and for distinguishing among different virus serotypes (serotyping). Due to the co-circulation of multiple serotypes of Bluetongue virus (BTV), the neutralization test has become an important surveillance method in Europe. However, the existence of different protocols makes test standardization and interpretation of results difficult. The current paper describes the development of a neutralization test using plasma and addresses the factors critical for detection of neutralizing antibodies against BTV serotype 8 (BTV-8), such as virus propagation, stability of virus infectivity and origin of the BTV-8 strain. The results indicated that animals exposed to the Northern European BTV-8 strain developed low neutralizing antibody titers, particularly after vaccination and experimental infection. Although clearly ELISA-positive, these samples often yielded false negative results when tested by the neutralization test using the OIE recommended virus concentration of 100 $TCID_{50}$/50 μl. The sensitivity of the neutralization test could be improved significantly with retained specificity by using a reduced $TCID_{50}$ and the homologous European BTV-8 strain instead of the South African reference strain.

1. Introduction

Bluetongue disease in ruminants is caused by Bluetongue virus (BTV), a member of the genus *Orbivirus*, family *Reoviridae* (Murphy et al., 1971). Within the BTV serogroup there are 26 immunologically distinct serotypes recognized worldwide, of which nine currently are known to circulate in Europe (Maan et al., 2011; Hofmann et al., 2008; Saegerman et al., 2008).

Viral protein (VP) 7 of the BTV particle is the group-specific core protein conserved among all BTV serotypes, whereas VP2, encoded by segment 2 of the BTV genome and located in the outer capsid layer, represents the main determinant of the serotype and the neutralization-specific immune response (Huismans and Erasmus, 1981). Within 7–14 days post-infection (dpi), animals develop both non-neutralizing and neutralizing anti-BTV antibodies (Stott et al., 1985) which are considered long-lasting after natural infection and immunization with live-attenuated vaccines. Although neutralizing antibody protects against infection with homologous virus, heterologous immunity also has been observed (Roy et al., 1994). In addition, anti-BTV cytotoxic T lymphocytes partially confer *in vivo* protection (Jeggo et al., 1985).

The majority of commercial ELISA kits detect all BTV serotypes, because they are designed to bind antibodies against VP7. Although segment 2-specific RT-PCR primers and assays have been developed (Eschbaumer et al., 2011; Mertens et al., 2007), neutralization tests are still the traditional approach to determine the BTV serotype (OIE, 2009). In this regard two types of neutralization test need to be distinguished: the virus neutralization test utilizes standard concentrations of a known serum to be tested with increasing dilutions of an unknown virus. For serotyping, a panel of sera raised against all recognized BTV serotypes is used for neutralization against the unknown virus (OIE, 2009). In contrast, the serum neutralization test determines the presence and titer of neutralizing antibodies of an unidentified serum sample against a known virus. In this case the *in vitro* neutralization of virus provides evidence of the level of *in vivo* protection against a homologous virus infection (Sundin et al., 1989) and is used to determine immune status after virus exposure.

Virus neutralization is based on complex formation of virus with neutralizing antibody and takes place *in vitro* during incubation. Infectivity of non-neutralized virus particles is visualized through subsequent inoculation of the virus–antibody mixture onto a sensitive *in vitro* system. Virus replication may be determined by

* Corresponding author at: Center for Vectorborne Diseases, Department of Pathology, Microbiology and Immunology, School of Veterinary Medicine, University of California, Davis, CA 95616, United States of America. Tel.: +1 530 304 0788; fax: +1 530 754 8124.
E-mail address: gworwa@ucdavis.edu (G. Worwa).
[1] Present address: Center for Vectorborne Diseases, Department of Pathology, Microbiology and Immunology, School of Veterinary Medicine, University of California, Davis, CA 95616, United States of America.

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observing cytopathic effect (CPE), immunofluorescent staining or plaque inhibition on inoculated cell cultures (OIE, 2009).

The recent BTV outbreaks caused by serotype 8 (BTV-8), the introduction and circulation of additional serotypes (Saegerman et al., 2008), the finding of Toggenburg Orbivirus (Hofmann et al., 2008), and the extensive vaccination against BTV in Europe emphasize the need for a reliable neutralization test protocol. However, incubation parameters, selection of virus strain, virus stock production and titer, virus–antibody concentration, and the sensitivity of the selected *in vitro* indicator system are factors that complicate the establishment of a standardized protocol and make the comparison of results among laboratories difficult. This problem was addressed by optimizing the neutralization test using plasma for the detection of neutralizing antibodies against BTV-8 based on a microneutralization assay with CPE detection on Vero cells.

2. Material and methods

2.1. Animals and sample collection

Animal experimentation was approved by the Cantonal veterinary services in Bern (approval #39-07) and animals were held according to the laws on care and use of laboratory animals in Switzerland.

2.1.1. Samples from naturally infected animals

Single plasma samples from 13 and 16 naturally infected cattle and sheep ($n = 29$), respectively, were collected during the 2007/08 BTV-8 outbreak in Switzerland.

2.1.2. Samples from experimentally infected animals

Plasma samples collected from 24 sheep between 0 and 16 dpi and from 3 sheep between 0 and 147 dpi were available from a previous experimental BTV-8 infection study ($n = 27$) (Worwa et al., 2010).

2.1.3. Samples from vaccinated animals

Single plasma samples from 58 cattle vaccinated with a monovalent inactivated BTV-8 vaccine (BTVPUR AlSap^{TM} 8, Merial, France) were taken after administration of two vaccine doses as specified by the manufacturer.

2.1.4. Samples from BTV-seronegative animals

Plasma samples from 39 cattle, 11 sheep, 6 goats and 4 llamas ($n = 60$) were tested seronegative for BTV using a Bluetongue Virus Antibody Test Kit, cELISA (VMRD, Pullman, USA).

2.1.5. Sample processing

Plasma was obtained from blood supplemented previously with EDTA after centrifugation at 1150 × *g* for 10 min at 4 °C. Prior to analysis, plasma was heat inactivated at 56 °C for 30 min followed by centrifugation as described above.

2.2. Cells

Vero cells (African green monkey kidney, ATCC, USA) were cultured in GMEM (GIBCO) supplemented with 10% fetal bovine serum (FBS, previously tested to be free from BTV, pestiviruses and anti-BTV antibody) in a humidified atmosphere at 37 °C and 5% CO_2 (standard conditions). Cells were split twice a week up to a maximal passage of 20, regularly analyzed for vitality using trypan blue staining (GIBCO), and tested to be free from mycoplasma contamination.

For BTV inoculation, cells were 70–80% subconfluent, 24 h old monolayers cultured in GMEM supplemented with 10% FBS, 1% penicillin/streptomycin and 0.25% amphotericin B.

2.3. Viruses

2.3.1. Virus isolates

The South African BTV-8 reference strain (BTV8_SA) (kindly provided by the Institute for Animal Health [IAH], Pirbright, UK) and the Northern European BTV-8 strain (BTV8_EU) (kindly provided by the Friedrich-Loeffler-Institute [FLI], Riems, Germany) were selected for stock virus production. BTV8_EU was used to infect animals experimentally as described in Section 2.1.2.

2.3.2. Virus stock production for BTV-8 neutralization test

2.3.2.1. Conventional production. Prior to infection, growth medium was removed and Vero cells were washed twice with FBS-free medium. BTV8_SA and BTV8_EU were inoculated onto cells at a 1:10 dilution and growth medium was added 1 h after incubation at standard conditions. Virus was harvested 4–6 dpi when approximately 70% CPE was present. Cell culture flasks were frozen, thawed, the suspension removed, and cell debris separated by centrifugation at 1150 × *g* for 20 min at 4 °C. The $TCID_{50}$/50 μl of each virus was determined by titrating 10-fold dilutions of the supernatant on Vero cells in 96-well flat-bottomed microtiter plates. The viruses were aliquoted and stored at −70 °C.

2.3.2.2. Modified production. The following parameters were modified for the propagation of BTV8_EU: a multiplicity of infection (MOI) of 0.1 was used for inoculation of Vero cells. Virus was harvested either at 5–10% CPE or within 24–48 h by removing the cells with a sterile cell scraper. The cell suspension was sonicated at high amplitude intensity (cell lysis 2.3 in Table 1) for 1 min using a high intensity ultrasonic processor (Sonic & Materials, USA), centrifuged as above and then stored at +4 °C. The amount of virus resulting in 100 $TCID_{50}$/50 μl and 20–40 $TCID_{50}$/50 μl was determined by titration on Vero cells.

2.3.3. Comparison of different production and storage methods for BTV8_EU

To determine the conditions at which BTV-8 was most stable over time, two approaches were used: first, the harvest technique that produced the greatest yield of infectious virus was determined, and secondly, different storage conditions were evaluated with respect to preservation of infectious virus titer. A stock of BTV8_EU was produced according to the method described in Section 2.3.2.1, from which a total of 20 different variants were generated (Table 1). For virus harvest, the infected cells were either lysed or supernatant was removed from flasks without disruption of cells (Table 1). Cell lysis was performed either by freeze–thaw of the cell culture flask at −70 °C (cell lysis 1.1 in Table 1) or by sonication at increasing amplitude intensity levels (cell lysis 2.1 at low, cell lysis 2.2 at medium and cell lysis 2.3 at high amplitude intensity) (Table 1). Aliquots of harvested virus variants were stored at +4 °C and −70 °C solely or supplemented with 99.5% sterile glycerol in a 1:2 dilution according to Table 1. Virus titers were determined after harvesting and after one year of storage by titrating 10-fold dilutions of the supernatant on Vero cells in 96-well flat-bottomed microtiter plates as described above.

2.4. Neutralization test

2.4.1. Virus concentration using high $TCID_{50}$ and low $TCID_{50}$

The $TCID_{50}$ represents the median tissue culture infection dose, or, the number of plaque forming units of virus estimated to cause CPE in 50% of infected cell cultures. Two-fold serial dilutions from