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Transplacental infection in goats experimentally infected with a European strain of bluetongue virus serotype 8

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ABSTRACT

The capability of the recently emerged European strain of bluetongue virus serotype 8 (BTV-8) to cross the ruminant placenta has been established in experimental and field studies in both sheep and cattle. Seroprevalence rates in goats in North-Western Europe were high during the recent outbreak of BTV-8; however the capability of the virus to infect goats through the transplacental route has not been established.

In the present study, four Saanen goats were inoculated with the European strain of BTV-8 at 62 days of gestation; this resulted in mild clinical signs, however gross lesions observed post mortem were more severe. Viral RNA was detected by real-time RT-PCR in blood and tissue samples from three fetuses harvested from two goats at 43 days post infection. Conventional RT-PCR and genome sequencing targeting viral segment 2 confirmed infection of brain tissue with BTV-8 in two of these fetuses. In total, five of six fetuses demonstrated lesions that may have been associated with transplacental infection with BTV. Infected fetuses did not demonstrate neurological lesions. Low viral RNA concentrations in fetal blood and tissue further suggest that the infected fetuses would probably not have been born viraemic. The implications of these findings with regards to the epidemiology and overwintering of BTV-8 in Europe remains unclear.

Introduction

Bluetongue (BT) is a globally important haemorrhagic disease of domestic and wild ruminants that is caused by the bluetongue virus (BTV) (*Orbivirus* genus, family *Reoviridae*) (Borden et al., 1971; Mertens et al., 2005). Twenty-six serotypes of the virus have been identified (Maan et al., 2007, 2011; Hofmann et al., 2008). Bluetongue virus (BTV) is transmitted primarily through the bites of haematophagous midges (*Culicoides* spp.; Du Toit, 1944). Its distribution is therefore closely linked to the distribution of vector-competent midge species and suitable climatic conditions (mainly latitudes between 45–50°N and 35°S).

The global distribution of BTV has changed dramatically over the last two decades, especially in Europe, where several strains and serotypes have caused outbreaks far north of the virus's traditional boundaries in Northern Africa and the Mediterranean basin (Purse et al., 2005). The most economically damaging of these outbreaks started in 2006, when a strain of BTV serotype 8 (BTV-8) was introduced through an unknown route into North-Western Europe. The virus spread widely and eventually affected over 55,000 holdings (2007–2008) (Maan et al., 2008; Velthuis et al., 2010).

Several characteristics of the outbreak of BTV-8 in North-Western Europe made it unusual. The virus was spread by Palaearctic midge species (Meiswinkel et al., 2008) and appeared to be highly virulent, causing clinical disease not only in sheep, but also in cattle and goats (Thiry et al., 2006; Dercksen et al., 2007; Dal Pozzo et al., 2009). Infection of pregnant sheep and cattle also resulted in a high incidence of abortions, still births and malformations in offspring, suggesting that the virus was capable of crossing the ruminant placenta (Wouda et al., 2008; Vercauteren et al., 2008; Desmecht et al., 2008). Prior to this BTV-8 outbreak, transplacental infection had generally only been associated with the vaccination of pregnant sheep and cattle with egg and cell culture adapted modified-live virus (MLV) (Kirkland and Hawkes, 2004), whereas wild-type strains were generally thought to be incapable of doing so.

Transplacental infection with the current European BTV-8 strain has been investigated in both sheep and cattle (Worwa

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et al., 2009; Backx et al., 2009; van der Sluijs et al., 2011; De Clercq et al., 2008; Darpel et al., 2009; Santman-Berends et al., 2010; Saegerman et al., 2010). Field surveys have indicated that the virus was very efficient at crossing the placenta; transmission rates of 33–41% were recorded in calves born to previously infected dams in The Netherlands, Belgium and the United Kingdom (Darpel et al., 2009; De Clercq et al., 2008; Desmecht et al., 2008). This high rate of transplacental transmission may have been a contributing factor that allowed the virus to persist during vector free or low European winters. Several studies have demonstrated the birth of seemingly healthy reverse transcriptase (RT) PCR positive and/or viraemic lambs or calves (De Clercq et al., 2008; Menzies et al., 2008; Santman-Berends et al., 2010; Saegerman et al., 2010). These animals may have served as a source of virus for newly emerged adult midges in subsequent vector seasons.

Goats were infected at a high prevalence during the BTV-8 outbreak in North-Western Europe. In 2007 the seroprevalence rate in The Netherlands was estimated at 47% (Elbers et al., 2008) and 25% in Germany (Conraths et al., 2009). Although transplacental infection of sheep and cattle with the BTV-8 strain has been demonstrated, no information is available regarding infection of goats by this route. The potential role of goats as overwintering hosts of BTV-8 therefore remains unclear. The present study was designed to investigate whether BTV-8 is able to cross the caprine placenta.

Materials and methods

Virus

BTV-8 isolated from a clinical bovine case (NET2007/01; IAH, Pirbright, UK) was used for infection. A virus stock for inoculation was prepared by passing the virus twice in African green monkey kidney (Vero) cells (E1/BHK2/V2). Virus containing supernatant was subsequently titrated in Vero cells and diluted in medium to a final titre of 4.75 $\log_{10}TCID_{50}$/mL (Reed and Muench, 1938). Vero cells were grown in 75 cm^2 flasks containing 30 mL Dulbecco's Minimal Essential Medium (DMEM) supplemented with 5% fetal calf serum (v/v), 10% tryptose phosphate broth (v/v) and 1 mg/mL gentamycin and incubated at 37 °C in an atmosphere containing 5% CO_2 until 90% confluent.

Animals

Pure-bred Saanen goats between 1 and 2 years of age were procured from a local source in Pretoria. The BTV serological status of the animals was evaluated by a cELISA (see below) and six seronegative healthy appearing animals selected. The animals were housed in open stables containing insect traps and routinely treated with a topical insect repellent (Cylence, Bayer). Oestrus was synchronized, after which the goats underwent intra-cervical insemination with fresh BTV real-time RT-PCR negative semen. Two non-viraemic and seronegative rams were left with the goats for 24 h to allow for natural service. Twenty-nine days later four pregnant animals (confirmed using ultrasonography) were selected for the challenge phase and the two non-pregnant goats included as negative controls. The animals were transported to an insect secure biosafety level-3 (BSL-3) holding facility (Onderstepoort Transboundary Animal Diseases Facility) where the inoculations were conducted. All animals were accommodated according to the guidelines of the University of Pretoria's Animal Use and Care Committee (AUCC project number V059-10).

Experimental design

On day 62 of gestation (study day 0) each pregnant goat was injected with 1 mL of BTV-8 inoculum IV and 1 mL SC on the side of the neck. Control animals were inoculated in a similar fashion with BTV negative cell culture medium. Following challenge, the goats were observed twice daily for clinical signs. BTV-8 inoculated goats were sequentially euthanased through the IV injection of sodium pentobarbitone (200 mg/kg) on day 13 (animal 1), 25 (animal 2) and 43 (animals 3 and 4) post infection (dpi). The control animals (animals 5 and 6) were euthanased at the end of the trial (46 dpi). All fetuses and adults were examined post mortem.

Sample collection

Blood samples (EDTA, heparin and serum) were collected from all goats prior to inoculation (day −14 and 0), daily for 21 dpi and weekly thereafter until the experimental endpoints. Skin samples were also taken from each animal on a weekly basis during the first 21 dpi and on the day of euthanasia. At necropsy, tissues (skin, brain, submandibular lymph node, tonsils, lung, spleen, kidney, liver, heart, tongue, mammary tissue, uterus, cotyledon, fetal membranes, umbilical cord) and blood samples were collected from adults and fetuses. Care was taken to avoid contamination of fetuses with maternal blood.

Competitive enzyme linked immunosorbent assay (cELISA)

Group-specific antibodies to BTV were measured in adult goats at different intervals and in serum from fetuses at necropsy using a cELISA (Veterinary Medical Research and Diagnostics) according to the manufacturer's instructions. Percentage negativity values were calculated by using the following formula: [1 − (OD sample/OD negative reference)] × 100 and a cut-off value of >50% used to distinguish between positive and negative sera.

Serum neutralization tests (SNT)

Each serum sample that tested positive in the cELISA was tested in duplicate for the presence and titre of neutralizing antibodies to BTV-8 using an SNT (OIE, 2004).

RNA extraction

For real-time RT-PCR total RNA was extracted from 100 μL EDTA stabilized blood or 100 mg of tissue (preserved in RNAlater, Ambion) by using Trizol (Invitrogen) according to the manufacturer's instructions and resuspended in 50 μL AVE buffer (QIAGEN). For conventional RT-PCR (sequencing), RNA was extracted from selected blood and tissue samples using Trizol. Viral double stranded RNA (dsRNA) was thereafter precipitated (Potgieter et al., 2009) and concentrated by passing several parallel samples through one QIAquickPCR purification column (QIAGEN) prior to washing and elution.

Real-time RT-PCR targeting segment 5

Real-time RT-PCR was conducted on RNA from blood and tissue samples by using a two-step procedure (Toussaint et al., 2007) targeting genome segment 5 (encodes non-structural protein 1, NS1). Briefly, dsRNA was denatured with 10% DMSO (v/v) at 95 °C for 3 min and snap cooled on ice prior to RT on 2 μL using the High Capacity RNA to cDNA master mix (Applied Biosystems) and 1.25 μM random hexamers (25 °C for 5 min, 42 °C for 30 min and 95 °C for 5 min). Real-time PCR was performed using the TaqMan Universal Master Mix II (Applied Biosystems) with 10 pmol of each of the primers BTV S5 F1-19 and BTV S5 R76-57, 2.5 pmol TaqMan probe (BTV S5 P 49-27) and 5 μL cDNA (20 μL in total). Cycling conditions were 95 °C for 10 min followed by 50 cycles at 95 °C for 15 s and 58 °C for 1 min. Mammalian beta-actin mRNA was amplified as an internal control using the same conditions. Samples were considered BTV positive if they demonstrated a cycle threshold (Ct) value <45.

Conventional RT-PCR and sequencing

Conventional PCR (Mertens et al., 2007) was conducted on enriched viral dsRNA from samples which had tested positive in the NS1 real-time RT-PCR. Briefly, dsRNA (5 μL) was denatured with 1 μL 0.2 M methyl mercuric hydroxide (MMOH) for 10 min at room temperature. One mL of 1.0 M 2-mercaptoethanol was added prior to RT on 5 μL using the High Capacity RNA to cDNA master mix (Applied Biosystems) and 10 pmol of each of two BTV-8 segment 2-specific primers (BTV-8/2/710F; BTV-8/2/897R) (25 °C for 5 min, 42 °C for 30 min and 95 °C for 5 min). Each 50 μL PCR reaction contained DreamTaq Green PCR Master Mix (Fermentas), 20 μL of cDNA and 10 pmol of each primer. The thermal profile was: 95 °C for 5 min; and 40 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 2.5 min, followed by 72 °C for 10 min. The PCR products were analyzed by 1% agarose gel electrophoresis and visualized under UV light after staining with ethidium bromide.

Amplicons of the correct size (~560 base pairs) were purified with a QIAquick PCR purification kit (QIAGEN) and sequenced using the V3.1 BigDye Terminator system (Applied Biosystems) and 2.5 pmol of either the reverse or forward primer. Unincorporated labelled ddNTPs were removed by ethanol–sodium acetate precipitation and the reactions resolved on an ABI 3100 DNA sequencer. Sequences were assembled and trimmed (giving 499 base pairs corresponding to position 2163–2661 of BTV-8) with the Staden package V4.1 (Bonfield et al., 1995) and were subjected to a nucleotide–nucleotide sequence BLAST on the National Centre for Biotechnology website.

Immunostaining

Immunostaining was performed on tissue samples preserved in 10% formalin buffered saline (Sanchez-Cordon et al., 2010) with a 1:200 dilution of in-house