Lack of detectable bluetongue virus in skin of seropositive cattle: implications for vertebrate overwintering of bluetongue virus

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Summary

The overwintering mechanism of bluetongue virus (BTV) has eluded researchers for many years. It was recently proposed that ovine gamma delta T-cells may become persistently infected with BTV, and serve as a reservoir for infection of naive vectors in the next transmission season. Since cattle are more numerous than sheep in the western United States (where BTV is endemic), this hypothesis was tested in bovines. In the winter of 2002-2003, 54 cattle from an endemic site in northern Colorado were age-selected to ensure that possible BTV exposure must have occurred in the summer of 2002. These cattle were tested for the presence of anti-BTV antibody by ELISA; 53 were seropositive, and one was seronegative. Naive *Culicoides sonorensis* colony insects were fed on skin sites of four seropositive and one seronegative cattle at day 1 (135 days after the first frost), then sequentially on separate sites for three days. Virus isolation and/or reverse transcriptase-nested polymerase chain reaction from engorged insects and 6 mm skin biopsy samples were performed for detection of viable BTV or BTV nucleic acid; all were negative. These data suggest that cattle are not a reservoir host for BTV overwintering in the western United States. The role of sheep in the trans-seasonality of BTV still remains to be determined.

Keywords

Bluetongue – Cattle – *Culicoides sonorensis* – Overwintering – United States of America – Virus.

Introduction

Bluetongue virus (BTV) is the prototype of the genus *Orbivirus*, family *Reoviridae*. BTV is an arthropod-borne virus that is maintained in the United States of America (USA) in a natural transmission cycle involving the primary haematophagous insect vector *Culicoides sonorensis* and sheep, cattle and wild ruminant amplification hosts (21, 41). The BTV genome is composed of ten double-stranded RNA segments designated large (L1-3), medium (M4-6), and small (S7-10) (41). In the mature virion, the segments are encapsidated in two concentric protein shells. The outer capsid is composed of virion proteins (VP) 2 and 5. The inner capsid is composed of VP7 trimers that 'float' dynamically on VP3 pentamers, which provide the scaffolding for viral assembly (20) and bind to the dsRNA. Genome segments S7 and L3 encode VP7 and VP3, respectively. VP7 is a group specific antigen (10), and other work has demonstrated that segments from multiple serotypes can be detected with a single primer set (51).

BTV overwintering/trans-seasonality

BTV may be maintained in year-round transmission cycles in tropical parts of the world, and even possibly in mild temperate winters (16; F. Holbrook, personal communication). However, such cycles are impossible in some regions due to environmental conditions (18). The virus must have a mechanism(s) that allows it to survive periods of inclement climatic conditions and vector diapause in temperate regions of the world, as well as in tropical regions that experience a significant dry season. Three principal hypotheses have been proposed for BTV trans-seasonal maintenance, as follows:

1) high altitude, air-current based, reintroduction of infected insects from year-round cycles to

endemic foci on a yearly basis

2) vertical transmission from the infected insect to its progeny
3) survival in the vertebrate host, either through persistent infection of adults or transplacental transmission to foetuses.

Annual reintroduction of bluetongue virus-infected culicoids to endemic foci in temperate climates

The reintroduction of infected insects from continuous, tropical transmission cycles to endemic foci in temperate zones is unlikely to account for the observed epidemiology of BTV. Several areas of the world do have low-level BTV activity year-round (16, 31), and introduction of BTV-infected culicoids into previously BTV-free, local areas on air currents can be an important mode of transmission on a small geographic scale (37). However, previous attempts to support this hypothesis (7, 42, 43, 44, 45) have failed to account for the random nature of aerial dispersal of pathogens (9) versus the epidemiological stability of BTV within endemic foci. In the absence of a consistent and positive link between weather patterns and the natural state of BTV in temperate zones, this hypothesis must be considered to be the least likely of the possibilities.

Overwintering of bluetongue virus in the invertebrate vector

The hypothesis that BTV overwinters through vertical transmission of the virus by infected adult *C. sonorensis* to their overwintering larval progeny is supported by our previous studies (14, 38, 51). BTV has been isolated from adult midges (reared in the laboratory from field-collected larvae) from an endemic focus in northern Colorado near the commencement of the transmission season (14), and BTV dsRNA and antigen have been detected in *C. sonorensis* larvae collected from the same focus (38, 51). Although experimental evidence indicated that transovarial transmission (TOT) of BTV does not occur in colonised *C. sonorensis* (25, 33), it is possible that outbred insects may be capable of vertical transmission, especially in light of the studies cited above, as well as the isolation of another orbivirus (Orungo virus) from wild-caught male mosquitoes in the Côte d'Ivoire (11). In addition, BTV antigen was detected in the vitelline membrane of infected, adult females and proteoid yolk bodies of their oocytes, but not in the ovarian tissue itself. This demonstration of 'dense' BTV antigen in the reproductive structures in which it was found (33) would suggest that vertical transmission could occur. Vertical transmission of flaviviruses in mosquitoes occurs via the micropyle as the egg passes through the oviducts during oviposition (40). A similar mechanism could function for vertical transmission of BTV in *C. sonorensis*.

Overwintering of bluetongue virus in vertebrate hosts

For many years it was assumed that vertebrate animals exhibiting a long-term viraemia following acute infection was the most likely mechanism for BTV trans-seasonality. However, infectious virus is >99% likely to be eliminated from infected cattle after 63 days (46), and viral RNA cannot be detected by reverse transcriptase-nested polymerase chain reaction (RT-nPCR) after 222 days (4). Between those times, samples are RT-nPCR-positive but virus isolation (VI) negative, and non-infectious virus or viral RNA probably exists in close association with (or inside) erythrocytes (32). Studies were conducted to determine if the bite of an uninfected insect could rescue virus that had eluded attempts at virus isolation. Naive *Culicoides sonorensis* were intrathoracically inoculated with cattle blood samples positive by RT-PCR for viral RNA, but negative for BTV by VI. Not only did those insects not become infected, they also did not transmit BTV to uninfected sheep upon taking blood-meals after an appropriate extrinsic incubation period (48).

Assuming that animals with a long-term 'viraemia' (as above) cannot directly infect vectors, they would then have to be able to infect other vertebrates that could then serve as the reservoir for naive vectors. Venereal transmission of BTV in the semen of infected males (bulls or rams) is unlikely because virus can only be detected in semen shortly after peak viraemia (3 to10 days post infection) and is limited to that time alone (30). In addition, excretion of BTV in semen of naturally infected bulls appears to be an extremely rare event, if it occurs at all (15, 47). Finally, heifers impregnated with BTV-infected semen seroconverted in only 4 of 9 experimental exposures, and none of the foetuses showed any pathology or sign of infection (5, 6). Viraemias in infected calves last as long as 20 weeks (by RT-PCR detection), but they were shown to be infective for naive *Culicoides sonorensis* for only two weeks (27). Thus, venereal and/or vertical infection of vertebrate hosts with BTV represents a theoretical trans-seasonal maintenance mechanism; however, it would not appear to be a mechanism that is active in the natural cycle of the virus.

Immunosuppression induced by drugs (hormones or other), pathogens (i.e. ovine lentivirus), or stress could be responsible for the establishment of persistent BTV infection in sheep (8) and as such could have implications in the trans-seasonal

maintenance of BTV. While this is an intriguing possibility, its importance in the year-to-year maintenance of the virus cannot be fully assessed with the available data. We do know that naive vectors cannot recover latent virus from RT-nPCR-positive but VI negative cattle blood (48). However, those cattle may not adequately represent a truly 'persistently infected', stressed animal. Other immunosuppressive factors in addition to the stress of the bite(s) and the immunosuppressive qualities of culicoid saliva (35, 36) could increase the time that a cow or bull was infectious to a naive, competent vector. Further research in this area may be warranted.

Finally, persistent infection of immunotolerant cattle by BTV was reported in 1975 (22, 26), but this could be an artefact of laboratory-passaged BTV (2, 17, 19, 28, 50; A.J. Luedke and T.E. Walton, personal communications). Recent evidence has suggested that ovine γδ T-cell lines may become persistently infected with a laboratory-adapted strain of BTV, and that virus production is increased in response to cell-cycle arrest or activation (49). It was hypothesised that these T-cells could become persistently infected *in vivo*, migrate to the intraepithelial space, and become activated in response to the bite of a naive vector. They would then shed virus that would be available to infect either that vector or subsequently biting vectors. However, in a test of this hypothesis, virus was not detected by standard techniques in skin samples, blood samples, or naive culicoids fed on these 'persistently infected' sheep. The only way virus was detected was by culturing the skin samples in the presence of human IL-2 for 7 days. These findings are very provocative, and prompted us to test this hypothesis in cattle, which are much more prevalent than sheep in the western USA and therefore are more likely to play a pivotal role in the trans-seasonality of BTV in the country. Additionally, the authors themselves suggest that cattle are more likely to become persistently infected than sheep (49).

Materials and methods

Fifty-four cattle, approximately 8 to 10 months of age, were identified from a long-term endemic site in northern Colorado in January 2003. Blood samples were collected from these animals and assayed for the presence of serum antibody to BTV by a competitive enzyme-linked immunosorbent assay (c-ELISA) developed at the United States Department of Agriculture-Agricultural Research Service-Arthropod-Borne Animal Diseases Research Laboratory (ABADRL) (29). Fifty-three of the tested cattle were positive for antibody to BTV (>75% inhibition by c-ELISA), indicating exposure in the previous transmission season (summer 2002). Four seropositive animals and the one seronegative animal were brought into the Large Animal Isolation Building at the ABADRL in Laramie, Wyoming, for exposure to naive *Culicoides sonorensis* from our colony and sample collection. Four sites were shaved on each side of the animal and assigned a letter identifier (Fig. 1). All sites were exposed to insects on day 1, which was 135 days after the first significant frost in Adams County (14 October, 2002). Then, two 6 mm punch biopsies were taken from each site (A-H) after a subcutaneous lidocaine block was performed. One biopsy was placed in formalin, and the other was split for virus isolation (into cell culture medium) and RNA extraction (snap frozen in liquid nitrogen). Sites B, C, and D were fed upon by naive colony insects on days 2, 3, and 4, respectively, and skin samples were taken as above each day.

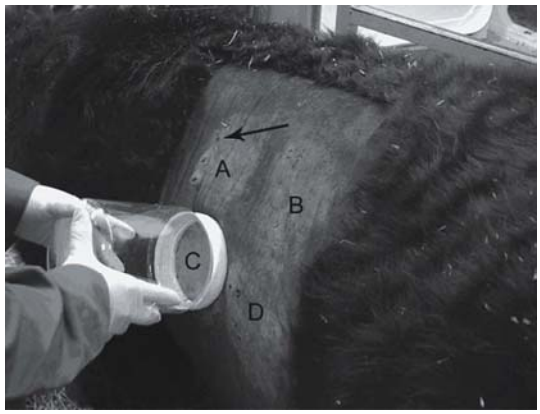

Figure 1
Experimental sites
Cattle were clipped in a large area, then closely shaved in the four areas where insects would be fed and skin samples would be collected (A-D)
The pattern was repeated on the opposite side of the animal as a negative control (no insect treatment, E-H)
Sites A-D were fed upon by naive *Culicoides sonorensis* on day 1, then sequentially on days 2 to 4 (i.e. site B was exposed to insects on day 2, site C on day 3, and site D on day 4)
Skin samples were taken with 6 mm punch biopsy instruments (arrow), and processed for virus isolation and detection of BTV nucleic acid by RT-nPCR

Skin samples for virus isolation were sonicated for 1 min in a cup horn sonifier (Ultrasonics Inc. Cell disruptor, Duty Cycle 60%, Output 6.5) in 0.5 ml of cell culture medium containing antibiotics (199-E, 10% foetal bovine serum, 400 IU/ml penicillin, 400 μg/ml streptomycin, 200 μg/ml gentamicin, 5 μg/ml amphotericin B) and directly plated onto Vero-MARU cells. Cultures were maintained for 10 days (3 to 4 days after positive controls showed 100% cytopathic effect). Cultures showing signs of bacterial or fungal infection were scraped and passed

through a 0.2 µm non-protein binding filter and placed onto a new flask of cells.

Insects were anaesthetised with carbon dioxide, and insects that had taken a blood-meal were removed for further processing (Fig. 2). Insects were pooled in 0.5 ml of cell culture medium containing antibiotics (as above) in groups of approximately 20 and ground using sterile microcentrifuge tube pestles. The ground insects were added to cells as above, held for 10 days, and processed in the same way as the skin samples.

Figure 2
Insect feeding cages
Insects were contained in cages with a fine mesh to allow for feeding on the animal
Insects were anaesthetised with carbon dioxide and blood-fed insects were removed and processed for virus isolation

Skin samples for nucleic acid detection were sonicated as above in 300 µl of cell lysis buffer from the Purescript kit (Gentra systems), held overnight at 4°C to allow full penetration of the lysis buffer, and processed according to the kit directions the next day. This protocol was found to be satisfactory after spiking extra skin samples from the seronegative animal with approximately 1 000 pfu of BTV-11 Station strain and comparing limiting dilutions of RNA extracted using the above sonication protocol, RNAlater-ICE treatment (Ambion Inc.), and grinding under liquid nitrogen with mortar and pestle.

Five microlitres of the extracted RNA were used as template for cDNA generation. Reverse transcription was performed using the Moloney murine leukaemia virus reverse transcriptase (MMLV-RT) (Promega Corporation), and 5 µl of the 20 µl reaction was used as template for the first round of PCR. After 35 cycles of 1 min at 95°C, 1 min at 56°C, and 2 min at 72°C (final extension of 10 min at 72°C), 5 µl of the first-round PCR was used as template for the nested PCR, which followed the same program. Primers for detection of the BTV S7 segment were designed from published sequences (Table I), and the same primers were used for both RT and the first round of PCR (23).

Table I
Primers used for the detection of the bluetongue virus S7 genome segment by RT-nPCR

Primer name	Primer sequence (5′->3′)*	Primer position
S7 forward	ATGGACACTATCGCTGCAAGAGCG	18
S7nest forward	GCCCGACCTCGTTGGCACAGAGAA	169
S7 reverse	CCCGCTTGAGTTTGCTGTGAATTA	614
S7nest reverse	GCGGCACGCATGAACCATCTCCCA	431

* All primers were designed based on published sequences

Results and discussion

All of the skin samples and blood-fed insects were negative for viable BTV isolation and/or BTV nucleic acid detection. RT-nPCR is the most sensitive detection method for bluetongue virus nucleic acid used today (3), and recovery of live virus by naive vector insects is recognised as the most sensitive and biologically relevant method of detecting latent BTV infections (48). The lack of detection of BTV by either method gives a high degree of confidence that the virus was not present in the skin of any of the animals.

Persistent infection of immunotolerant cattle by BTV was suggested as a virus overwintering mechanism (22, 26), but these initial studies must be questioned because numerous attempts to replicate them have failed (2, 17, 19, 28, 34, 39, 50; T.D. St George and N.J. MacLachlan, personal communications). Perhaps the initial study was an artefact of cell culture passage of BTV (1; A.J. Luedke and T.E. Walton, personal communications). Other studies have suggested that the biological behaviour of laboratory-passaged BTV is different from virus that exists in nature. Laboratory-passaged and vaccine strains of BTV were shown to cause abortions in susceptible sheep, whereas wild virus did not (24, 47; T.D. St George, personal communication). Additionally, bulls with naturally acquired infections do not shed BTV in their semen, while only older bulls experimentally infected with laboratory-passaged virus rarely shed virus in the peri-viraemic period (15, 30, 47). Therefore, it would seem that any examination of the behaviour of bluetongue virus relevant to its natural epidemiology would require significant steps to ensure the 'wildness' of the virus.

The hypothesis proposed by Takamatsu *et al.* (49) is intriguing. However, their results could be an

artefact of virus '…passaged several times in BHK cells…', and have little relevance to the natural epidemiology of BTV. This view is supported by their failure to recover live virus from their persistently infected sheep by naive vector insect bite, as well as the failure to isolate viable BTV from the blood or skin samples that were processed normally. Only by culturing skin samples with human interleukin-2 for one week could virus be isolated. Other studies have indicated that IL-2 levels are either down-regulated (12) or not changed (13) in the inflammatory lesions generated by insect bites. However, other factors noted by the authors could be responsible for the activation of persistently infected γδ T-cells in the inflammatory lesions generated by the bite of vector insects (49).

As stated earlier (49), cattle, instead of sheep, are the more likely host in which one would anticipate the persistence of BTV. However, viable BTV and BTV-specific nucleic acids were not detected in BTV-exposed animals from an endemic focus in northern Colorado subsequent to feeding by naive vector insects. The role of ovines in the overwintering of BTV still remains to be resolved.

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