

A comparison of laboratory and 'wild' strains of bluetongue virus – is there any difference and does it matter?

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Summary

Bluetongue (BT) viruses (BTV) have been propagated in laboratory culture systems for more than 50 years, especially for the production of vaccines. An important outcome of the adaptation of these viruses to laboratory culture is attenuation of their virulence. As a consequence of this modification, it has been possible to produce vaccines that have reduced the clinical impact of BT in a number of countries. Unfortunately, the adaptation of these viruses has also introduced undesirable properties. In particular, modified live BT vaccine viruses have a high capacity to cross the ovine placenta and cause congenital abnormalities in the foetus. Modified strains of BTV have also been found in the semen of bulls and rams. It is possible that there are also other undesirable properties, including the potential to infect non-ruminant hosts. Because these characteristics are not properties of naturally occurring BTVs, the use of laboratory-adapted strains is not recommended when the biological properties of BTV are being studied.

Keywords

Attenuation – Bluetongue – Cell culture – Laboratory modified – Foetal defect – Semen contamination – Virus.

Introduction

The development of laboratory-based systems for the investigation of animal diseases such as bluetongue (BT) has been crucial to our understanding of the infectious agent and the disease process. Without the use of a wide range of laboratory tools, our knowledge would be very limited and research would progress very slowly. As a result of the adaptation of BT viruses (BTV) to grow in laboratory systems such as cell culture and embryonated chicken eggs (ECE), it has been possible to develop a range of diagnostic tests and vaccines. Studies of the structure of the virus, virus replication, interactions between virus and cells and other aspects of the basic biology of BTV have all been possible. The information gained from these studies has allowed the development of sophisticated molecular diagnostic procedures and the production of virus-like particles using recombinant DNA technology. Such advances would not have been possible if it was necessary to rely on virus that was amplified in its natural ruminant hosts.

An important consideration during the amplification of BTV in laboratory systems such as cell culture or ECE is that the progeny virus should retain the main characteristics that are representative of the parent virus. For both diagnostic tests and vaccines, the antigenic characteristics of the virus should be altered as little as possible. On the other hand, for vaccine production, there is a deliberate attempt to remove undesirable traits, especially the ability of BTV to cause disease in sheep. During the adaptation of the virus to a laboratory system and any modification to reduce the virulence of the virus (attenuation), it is desirable that no other change should occur to the biological or antigenic characteristics of the virus. For BTVs, it is probable that there have in fact been undesirable changes to the biological characteristics of these viruses. The purpose of this presentation is to review the adaptation of BTVs to laboratory systems, to present evidence of undesirable characteristics and to discuss the implications of these features.

Diagnostic reagents

During the development of diagnostic tests (other than virus detection by animal inoculation), there is no practical alternative to the use of BTV that has been amplified in a laboratory system such as cell culture or ECE. The main consideration of virus that is used for the production of diagnostic antigens is that the virus is faithfully representative of its serotype. During the passaging of viruses used for the production of diagnostic antigens, any significant deviation in antigenicity may be limited by the use of a seed lot system and by restricting the number of times that a seed is amplified. Variations in antigenic properties of the virus may be monitored by testing of the progeny virus against one or more type-specific reference antisera. Generally there are no changes of significance to virus used in this way for the production of diagnostic antigens.

Vaccines

Bluetongue was first described as a disease more than a century ago (24). It was feared as a major epidemic disease and consequently placed on the OIE List 'A'. Despite this ranking, and many reports of disease incidents to the OIE, there have been remarkably few outbreaks of BT described in the scientific literature. The situation with the development of vaccines presents a marked contrast. There are many reports of attempts to develop vaccines for BT. The earliest vaccines were developed in South Africa and were delivered as blood from infected animals. For about 40 years from 1907, strains of low initial virulence were utilised, maintained solely by passage in sheep (57). Deficiencies in these vaccines (occasional severe disease, and vaccine failure due to infections with other serotypes) led to their replacement.

Laboratory culture systems have since been used to produce a large volume of virus of known quality. Both live and inactivated vaccines have been evaluated. Although inactivated vaccines are very safe, they have generally been less effective and more expensive than live vaccines and have not been used on a commercial scale. In many respects, antigens for inactivated BTV vaccines are similar to antigen used for diagnostic reagents. Limits on the extent of repeated passaging and the use of seed stocks presumably restricts the occurrence of major antigenic changes. Provided steps are taken to maintain appropriate antigenicity, there can be no deleterious effects arising from the field use of inactivated vaccine because there is no real potential for the introduction of undesirable genetic material into the environment. While not proven, it is also

unlikely that other non-infectious vaccines (e.g. sub-unit, virus-like particles or possibly DNA vaccines) will transfer unwanted genetic material to mammalian hosts. The situation with viruses used to produce live vaccines may, however, be quite different.

Modified live vaccines and attenuation

The biological characteristics of BTVs are extremely complex. The BTV serogroup is relatively large and, although all of the viruses, by definition, share certain features, there is also considerable diversity. Shared antigenic characteristics unite these viruses, but the assignment of a virus to a serotype also denotes that there are differences between members of the group. Interestingly, the elements that define serotype are not directly linked to those that influence perhaps the most important elements – the determinants of pathogenicity and virulence. Within a serotype, there can be virus strains that are highly pathogenic and others that, at best, cause very mild disease. For example, strains of BTV-1 in South Africa or the People's Republic of China have caused large disease outbreaks while there are Australian serotype 1 viruses that are non-pathogenic.

There have been two main considerations during the development of live BT vaccines. These have been safety and efficacy. Safety considerations have predominantly focused on ensuring that viruses that are amplified for vaccine production do not cause disease, or, at worst, that the clinical signs that occur are very mild. A secondary consideration has been the reversion to virulence, based on concerns that a modified virus may resume virulence characteristics after repeated passage in mammalian hosts. During attempts to reduce the virulence of a 'wild-type' virus by manipulation in laboratory systems, there has often been a delicate balance between achieving an acceptable degree of attenuation and maintaining an appropriate level of immunogenicity. Even today, at a molecular level, the basis for attenuation is still poorly understood and the outcome of attempts to modify a virus cannot be precisely controlled.

There have been attempts to attenuate BTVs for more than 50 years (3). Two main systems have been utilised, namely: propagation in ECE and in cell cultures of various types. Adaptation of a virus to growth in ECE or cell culture induces desirable changes that result in attenuation. BTV adapted to growth in ECE and repeatedly passaged to achieve an appropriate reduction in virulence was used for vaccine production for several decades in South Africa (3, 21). Once cell cultures became available, these were also used to serially propagate viruses. Both ECE and cell culture propagated vaccines were

used concurrently in South Africa (23), although little data has been published on their safety and efficacy. In 1952, BTV was officially recognised in the United States of America (USA), and vaccines were developed. Importation of vaccines from South Africa was not permitted. However, vaccines based on ECE propagation of USA strains (32) were manufactured locally. Later, high passage BTV that had been propagated in ECE was used as the starting material for further passage in cell culture (27). More recently, candidate USA vaccines of solely cell culture derivation have been produced and evaluated (31). These vaccines have been beneficial and have significantly reduced the impact of BTV infection in countries where they have been used. Other countries have also developed modified live vaccines after propagation in ECE or cell culture (63).

Mechanism of virus attenuation

The molecular basis by which attenuation of BTV strains is achieved is not clear. Furthermore, there are no precise criteria that have been followed to uniformly achieve the required end result of a vaccine virus with minimal virulence but optimal immunogenicity. Nevertheless, there are some trends in practices that have been adopted to develop modified live vaccines. Most vaccines have been based on seeds that are the product of repeated passage of virus in laboratory culture systems, usually involving cells of species different from the target mammalian host. As there is considerable variation in the virulence of field strains, it is not unexpected that there have been different levels of passaging required to achieve suitable attenuation. However, the passage level for ECE-adapted viruses has usually ranged from 30 to 68, with earlier passage levels producing unacceptable reactions in vaccinated animals (3, 32, 37, 66). There is also some evidence, that excessive passage in ECE may lead to over-attenuation of the virus (66). The temperature of incubation of the eggs also affects the passage level at which optimal attenuation occurs (3). In one study (15) a single passage in ECE, followed by another single passage in cell culture, was sufficient to achieve attenuation. In other situations, more extensive passaging in combinations of host systems has been followed (13, 27). Adequate attenuation of Australian BTVs has been achieved after about 20 passages in BHK-21 cells (30, 63) but 'wild' strains of these viruses are generally of lower virulence than the same serotypes in South Africa (18).

There have been few studies to identify the determinants of virulence and mechanisms of attenuation of BTV at the molecular level. There are some indications that genome segments 2 (51) or 2

and 6 (22) might be involved, but sequencing studies did not support this (15). The most convincing work to date has involved mouse-adapted variants of USA serotype 11 (UC-2 and UC-8) in a model system involving new-born mice and in subsequent studies in cattle. These studies indicated that segment 5 of the genome was associated with virulence (58, 59, 60, 61, 62).

Foetal infections and teratogenicity

While it has been possible to achieve desirable modifications to a range of different BTVs, a key issue is whether there have been any adverse outcomes arising from the amplification of BTVs in laboratory systems. Unfortunately, the passage of BTVs in ECE or cell culture can induce undesirable properties. Some of these changes appear to occur after relatively limited manipulation in laboratory systems. One of the most prominent features of laboratory-adapted virus (for example, some attenuated or modified live vaccine viruses) is the ability of the virus to cross the placenta, causing foetal abnormalities, abortion and perhaps other reproductive losses. Concerns about the teratogenicity of attenuated BTV vaccines first arose following the use of an ECE-adapted vaccine (55). The teratogenic effects of modified live vaccines for BTV are now well recognised (39) and vaccination of pregnant ewes is contraindicated.

Natural infection of sheep and cattle with 'wild' strains of bluetongue virus

Foetal infection following natural exposure of sheep, cattle or goats to 'wild-type' strains of BTV seems to be a very rare occurrence. Sometimes abortion has occurred in sheep after infection with pathogenic strains of BTV, but this has been considered to be secondary to the febrile illness affecting the ewe. In countries where live vaccines have not been used, there is no evidence of virus crossing the placenta. For example, in Australia, in some years up to 0.5 million cattle may be infected with a strain of BTV, without adverse sequelae.

Experimental infection of sheep with 'wild' strains of bluetongue virus

When sheep have been infected experimentally with virus that has been derived directly from the field, and has not been knowingly passaged in a laboratory culture system, there are some apparently conflicting results. The inoculum used for such studies has been blood that contains virus that has been maintained by repeated passage in sheep or cattle. It has always been assumed that repeated passage between mammalian hosts, without a cycle through the arthropod vector, does not alter the virulence of the virus and presumptively does not alter the

characteristics of the virus. In studies in the USA (4, 8), clinical signs were observed in a high proportion of ewes that were inoculated with unadapted virus. The virus did cross the placenta and caused foetal death in up to 40% of lambs (4). Another study in Cyprus (49) compared both laboratory-adapted and field strains. Both strains crossed the placenta, infected the foetus and caused lesions in a range of organs including the brain. These results are in marked contrast to those of three similar studies, two of which were conducted in Australia (25, 26) and one in the USA (45). In each instance, ewes of similar stages of pregnancy (ranging from 25 to 45 days) were infected and many of the ewes showed signs of BT. However, there was either no evidence of foetal infection (25) or a very low (<5%) incidence of abortion and occasional isolation of BTV from foetal or placental specimens.

Infection of sheep with laboratory-adapted viruses

Since the early reports (9, 16, 54, 55) that described a significant incidence of foetal infections and congenital defects in lambs following vaccination of ewes with modified live vaccines, a number of experimental studies have been conducted (12, 26, 49, 65). These investigations were performed in several countries and used either ECE or cell culture adapted virus and studied a number of different serotypes of BTV. There were similar results and agreement that laboratory-adapted virus could readily cross the placenta and infect the foetus, with devastating results. Congenital defects, especially hydranencephaly, were consistently observed. The peak period of susceptibility was around 35-42 days of gestation.

Infection of cattle with laboratory-adapted viruses

Unlike the situation with sheep, there have only been a few studies of experimental infections of pregnant cattle with laboratory-adapted BTVs. Cattle were infected at several different stages of gestation, at times when a teratogenic agent would be likely to infect a foetus. There were no reports of adverse effects, in particular any evidence of transplacental infection, in any of these studies (44, 46, 50). There have been other studies that involved direct inoculation of the foetus *in utero* but these have not been considered in this review as they artificially bypass the placental barrier.

Bluetongue virus in semen

Another of the well-known properties of BTV that has had a profound impact on trade between countries is the excretion of virus in semen. Concern arose because of studies that suggested that some bulls may undergo a persistent infection and intermittently excrete virus in their semen (29). The

results of these studies are now of doubtful significance (33, 41). Nevertheless, there are many reports of studies of the testing for BTV in the semen of both naturally and experimentally infected bulls.

Natural infections of bulls

In both Australia and the USA, a large number of semen samples have been collected commercially from known seropositive bulls (28, 35, 47, 53). These represent mature bulls that have been infected with a range of different serotypes. BTV has not been isolated from any of these samples. Prospective studies (14, 34, 36) of the monitoring of sentinel bulls over periods of up to 15 years have also been described. Both blood and semen samples were collected regularly to monitor the occurrence of BTV viraemia and to detect BTV in semen. Over the time span of those studies, bulls were naturally infected with five different serotypes of BTV. There was only one possible infection of semen in one bull during the period of viraemia, but it was thought that this was probably an artefactual finding (36).

Experimental infection of bulls

With intense interest in the possible excretion of BTV in the semen of bulls and its potential ramifications for international trade, there have been many investigations of experimentally infected bulls (5, 6, 17, 19, 20, 28, 35, 42, 43). Unfortunately, parameters such as the age of the bulls or the passage history of the viruses have not always been clearly documented (6, 19, 43). Many of these studies appear to have utilised laboratory-adapted strains of BTV. It is now recognised that these factors may contribute to the possibility of BTV being detected in the semen (5). Consequently, a more recent study was specifically designed with some of these issues in mind (28, 35).

Collectively these studies of experimentally infected bulls have shown that virus may be found in semen only when it is also present in the blood or during the period of viraemia. Virus was detected in semen intermittently and only in the semen of a proportion of bulls, even though all animals became viraemic.

Apart from the investigation of natural infections, there is little data available with which to systematically compare the potential for laboratory-adapted and field strains of BTV to traverse the reproductive tract of bulls and contaminate the semen. There is only one extensive study in which both 'wild' and laboratory-adapted viruses (each from two serotypes) were compared concurrently and in both young and old bulls (28, 35). Virus was never detected in the semen of young bulls, whether infected with 'wild' strains of BTV or with cell

culture adapted viruses. In comparison, virus was found in the semen of many of the old bulls infected with cell culture adapted virus. Virus was also detected in the semen of some of the bulls infected with one of the 'wild' serotypes. However, these bulls were quite old (10 to 12 years) and blood was generally detected in the semen at the same time. It is believed that virus may be present in the semen of old bulls as a result of inflammatory changes that occur in older animals (5, 6) or if there is detectable blood in the semen (28, 35) perhaps also as a result of damage to the reproductive tract. Collectively, these studies suggest that laboratory-adapted strains of BTV are often found in semen, whereas 'wild' strains are infrequently found in semen, and only during the period of viraemia in old bulls.

Infection of rams

There is little published data on the infection of rams with BTV. However, in a limited study, laboratory-modified vaccine virus was detected intermittently in the semen of six-year-old rams during the period of viraemia, but not in the semen of young rams (S. Johnson, personal communication).

Bluetongue virus in non-ruminant species

BTV is traditionally considered to be a virus that infects ruminants but it can be adapted by laboratory modification to replicate in cells of other hosts, and *in vivo* in non-ruminant species (60, 61, 62). This was exemplified unexpectedly in 1993, when several cases of severe and fatal disease, characterised by pulmonary oedema, occurred in bitches that were in the late stages of pregnancy (1, 7, 11, 64). There were a large number of abortions but there was minimal evidence of viral replication observed in the placentae, and none in the aborted fetuses (7). BTV-11 was conclusively found to be the cause of the problem. All of the dogs had been vaccinated with a modified live multivalent canine vaccine and circumstantial evidence pointed strongly to the vaccine as the source of the BTV-11. Contamination of the cell culture used to produce the vaccine and/or the foetal calf serum used in the medium was suggested as a possible cause of the contamination. Following this incident, extensive serosurveillance of carnivores in Africa was performed to determine whether carnivore species in their natural environment (2) had been infected with BTV. Neutralisation tests indicated that many species had been infected naturally, and the route was conjectured as being oral, as a result of eating naturally infected ruminants. While there is no direct proof, there is speculation that the disease in the dogs was also a result of infection with a laboratory-adapted BTV.

Discussion and conclusions

It is clear from the numerous studies that have been undertaken that strains of BTV that have undergone adaptation to laboratory culture systems (either ECE or cell culture) have different biological characteristics to true 'wild-type' viruses. Attenuation of the virulence of wild strains has been the basis of the development of many effective vaccines for the control of BT. However, there is a similar body of evidence that shows that laboratory-adapted strains of BTV acquire undesirable properties, in particular the ability to cause foetal abnormalities, or excretion (or at least presence) of the virus in semen and perhaps a capacity to cause disease in non-ruminant species. Whether these undesirable traits are exclusively confined to laboratory-adapted viruses is not definite but it is very apparent that these features are markedly more pronounced in laboratory-modified strains of BTV compared to 'wild' strains.

Are there any other implications arising from these undesirable properties of laboratory-adapted virus? For simplicity and convenience, it has been usual practice to undertake studies of BTV with viruses that have been passaged in laboratory systems, mainly cell cultures. As there appear to be profound differences between cell culture passaged virus and true 'wild' strains, any *in vivo* studies of the basic biology of BTVs must include a parallel study of animals infected with virus that has not been adapted to laboratory systems. However, in some countries, there may be concerns that a BTV acquired from the field could contain genes from a laboratory-adapted virus. There are a number of documented examples of reassortment occurring between field viruses (10, 38, 52, 56). It is also probable that vaccine viruses have been spread by insect vectors. While it has been suggested that the very low titre viraemias of some vaccine viruses prevent transmission by vectors, this is somewhat contentious. There is evidence that some Australian field viruses (e.g. BTV-1) produce very low titre viraemias (less than $2.5 \log_{10}/\text{ml}$ of blood) in cattle (C.F. Williams and P.D. Kirkland, unpublished observations) but are transmitted widely by *Culicoides brevitarsis*, a relatively inefficient vector. During infection of either the arthropod vector or vertebrate hosts, vaccine viruses may have reassorted with true field strains (40, 48) and may have persisted in the field for many years (40). These reassortant viruses have effectively become modified field strains and it may now be difficult to acquire true 'wild' strains under these circumstances. If laboratory-adapted viruses, or reassortant viruses containing genes from laboratory-modified viruses are used in studies of the basic biology of BTVs, it is quite possible that the results may not be indicative

of natural BTV infection and incorrect conclusions may be drawn.

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