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Generation of Replication-Defective Virus-Based Vaccines That Confer Full Protection in Sheep against Virulent Bluetongue Virus Challenge▽

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The reverse genetics technology for bluetongue virus (BTV) has been used in combination with complementing cell lines to recover defective BTV-1 mutants. To generate a potential disabled infectious single cycle (DISC) vaccine strain, we used a reverse genetics system to rescue defective virus strains with large deletions in an essential BTV gene that encodes the VP6 protein (segment S9) of the internal core. Four VP6-deficient BTV-1 mutants were generated by using a complementing cell line that provided the VP6 protein *in trans*. Characterization of the growth properties of mutant viruses showed that each mutant has the necessary characteristics for a potential vaccine strain: (i) viral protein expression in noncomplementing mammalian cells, (ii) no infectious virus generated in noncomplementing cells, and (iii) efficient replication in the complementing VP6 cell line. Further, a defective BTV-8 strain was made by reassorting the two RNA segments that encode the two outer capsid proteins (VP2 and VP5) of a highly pathogenic BTV-8 with the remaining eight RNA segments of one of the BTV-1 DISC viruses. The protective capabilities of BTV-1 and BTV-8 DISC viruses were assessed in sheep by challenge with specific virulent strains using several assay systems. The data obtained from these studies demonstrated that the DISC viruses are highly protective and could offer a promising alternative to the currently available attenuated and killed virus vaccines and are also compliant as DIVA (differentiating infected from vaccinated animals) vaccines.

In the past decade, reverse genetics technology has revolutionized the science of negative-strand RNA viruses. Through the introduction of defined mutations into the viral genomes, remarkable progress has been made in our understanding of the pathogenicity and host range of these viruses, and this has provided the tools to produce rationally attenuated vaccines (5, 12). In contrast, reverse genetics for the double-stranded RNA (dsRNA) viruses of the family *Reoviridae* is in its infancy and, until recent years, the introduction of plasmid-derived sequence into the dsRNA genomes for this group of viruses has not been possible.

Bluetongue virus (BTV) belongs to the *Orbivirus* genus of the *Reoviridae* family. BTV is an insect-vectored emerging pathogen of wild and domestic ruminants and has recently had a severe economic impact on European agriculture. BTV causes disease in sheep, goats, and cattle, with mortality reaching up to 70% in some breeds of sheep (7). The disease is endemic in many tropical and subtropical countries. However, since 1998 incursions of BTV into mainland Europe have been common events, reaching as far north as Norway. Molecular epidemiology studies show that of the 24 serotypes, eight different serotypes (BTV-1, -2, -4, -6, -8, -9, -11, and -16) have been introduced into mainland Europe since 1998. Of these, BTV-8 caused the most severe disease in northern European sheep and cattle (15, 19, 22). The enlarged distribution of the *Culicoides* insect vector populations and virus transmission by novel species of *Culicoides*, which are abundant in central and northern Europe, have allowed the geographic range of BTV to remain extended into Europe (10). Thus, BTV now represents an ongoing threat to livestock in all European countries.

The virion is a nonenveloped icosahedral particle composed of three concentric protein layers enclosing a segmented genome of 10 linear dsRNA molecules (26, 37). The outer capsid, composed of two major structural proteins (VP2 and VP5), is involved in cell attachment and membrane penetration during the initial stages of infection (25, 27). The attachment protein, VP2, is also the serotype determinant of BTV and is the most variable viral protein. After entry, the outer layer of the virion particle is uncoated prior to the release of the remaining core particle from an early endosome into the cytoplasm of the host cell (8, 25, 27).

The core particle is made up of two concentric protein layers: the surface VP7 layer and an underlying VP3 layer which surrounds the genomic dsRNA segments, together with three enzymatic minor proteins, VP1, VP4, and VP6 (27, 31). The released core particle is transcriptionally active, synthesizing and extruding multiple capped positive-sense copies of each viral genome segment into the host cell cytoplasm (27). These transcripts have the dual roles of encoding the 11 viral proteins (seven structural and four nonstructural) and serving as templates for the synthesis of the new viral dsRNA segments (25, 27). Viral gene expression in BTV-infected cells relies on the

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viral transcripts produced by the infecting virus particle until the time when progeny core particles are assembled, which add to the pool of transcribing cores. Thus, we envisaged that if the viral single-stranded RNAs (ssRNAs) were delivered to the host cell cytoplasm by transfection rather than transcription from an infectious viral core, both functions of the ssRNAs would be achieved. A reverse genetics technology based on the recovery of virus from 10 plasmid-derived T7 transcripts has allowed the recovery of targeted mutants in a consistent genetic background (2). This has been extended by the use of complementing cell lines to enable defective mutants to be recovered (6, 17). Although the system has allowed the study of BTV in the context of a replicating virus, its use as a tool for generating specifically modified vaccines for BTV can address the need for vaccines that are both nontransmissible and effective. Early vaccines against BTV were attenuated virus strains; however, the use of these vaccines to control the disease has had a number of unwanted consequences, including teratological effects in newborn animals when pregnant sheep and cattle were vaccinated and reversion of the vaccine strains or reassortment between vaccine and field strains, leading to the generation of virulent strains (11, 20, 29). The serotype determinant for the virus (VP2) has been shown to be protective in sheep, as have virus-like particles (VLPs) produced by coexpression of the four structural proteins of the viral capsid (9, 30, 33). However, no commercial recombinant subunit BTV vaccine is currently available.

A functioning virus recovered from cloned genes should facilitate the rapid generation of defined attenuated vaccine strains. One class of these are DISC (disabled infectious single cycle) viruses, which replicate only once in normal cells due to the lack of an essential gene product but could still trigger both the neutralizing antibody response and an innate immune response through replication in the natural target cells. When the essential gene is deleted, such viruses are incapable of producing infectious virions unless the deleted function is provided in *trans* by a complementing cell line. To this end, we first generated a series of BTV-1 mutant viruses targeting VP6, an essential protein, and then examined their capacity to replicate both in wild-type cells and in a VP6-expressing cell line. These recombinant viruses produced no infectious virus in the wild-type cells due to the lack of VP6. However, each recombinant was infectious in the complementing cell line and yielded high numbers of infectious particles. In addition, we generated a reassortant DISC virus that consisted of the BTV-1 genetic background with the outer capsid proteins, VP2 (serotype determinant) and VP5 of BTV-8 (BTV-1/8D1), a highly pathogenic serotype, thereby altering the serotype to type 8. We examined whether these defective BTV-1 and BTV-1/8D1 DISC virus strains could elicit antibodies that could protect sheep against virulent virus challenges. The data obtained from vaccination trails demonstrated that indeed these DISC vaccine strains did not replicate in the animal hosts as expected but induced neutralizing antibodies that fully protected against the respective virus challenges.

MATERIALS AND METHODS

Cell lines and virus. BSR cells (BHK-21 subclone) were maintained in Dulbecco modified Eagle medium (DMEM; Sigma) supplemented with 4% (vol/vol) fetal bovine serum (FBS; Invitrogen). The stable BSR-VP6 cell line was grown in DMEM–4% FBS supplemented with 7.5 μg/ml of puromycin (Sigma)/ml. BTV serotype 1 (BTV-1) stock was obtained by infecting BSR cells at a multiplicity of infection (MOI) 0.1 and harvested 3 to 4 days postinfection.

BTV-1 and BTV-8 virus stocks were obtained by infecting BSR cells at a low MOI and harvested when a 100% cytopathic effect was evident. A series of VP6-deficient BTV stocks were obtained from the complementary cell line, BSR-VP6 cells, as described previously (17). Virus stocks were grown in BSR-VP6 and kept at low passage number (<5) for all experiments. Titers of viral stocks were obtained by plaque assay and are expressed as PFU per ml. Viral stocks were stored at 4°C. The preparation of dsRNA and purification of BTV core particles were as described previously (2, 3, 18, 36).

T7 plasmids for BTV transcripts and modified S9 transcripts. T7 plasmids for BTV transcripts and chimeric S9-EGFP transcripts used in the reverse genetics system were as described previously (2). Briefly, cDNA amplified from each segment was inserted into pUC19 (Fermentas) at the SmaI site with the T7 promoter at the 5′ end and a unique restriction enzyme site at the 3′ end (2, 4). T7 plasmids for BTV-8 S2 and S5 transcripts were constructed according to a protocol similar to that described by Boyce et al. (2). Modification of S9 and chimeric S9-EGFP was generated by using the available restriction sites in the S9 sequence of the T7 plasmid of BTV-10 S9 and chimeric S9-EGFP, and the sequence of each modified T7 plasmid was confirmed. Modification of BTV-1 S9 was generated by site-directed mutagenesis according to the method of Weiner et al. (38).

Synthesis and transfection of BTV transcripts. The synthesis of capped and uncapped BTV transcripts was as described previously (2, 4). All capped T7 transcripts were synthesized by using an mMESSAGE mMACHINE T7 Ultra Kit (Ambion) according to the manufacturer's procedure. For the synthesis of uncapped T7 transcripts, the RiboMAX Large-Scale RNA Production System T7 (Promega) was used according to the manufacturer's procedure. The synthesized RNA transcripts were dissolved in nuclease-free water and stored at −80°C. Confluent monolayers of BSR-VP6 were transfected twice with BTV mRNAs using Lipofectamine 2000 reagent (Invitrogen) as described previously (17).

Virus growth kinetics. For the growth curves of the mutant or control viruses, monolayers of BSR cells or BSR-VP6 cells were infected at an MOI of 0.01. At 0, 2, 24, 48, and 96 h postinfection (as indicated), the cells and supernatant were harvested together and disrupted by two freeze-thaw cycles, and the total titer was determined by plaque assay.

Ultrastructural analysis. BSR cells or BSR-VP6 cells were infected, and at 24 h postinfection they were processed for cell sectioning. Briefly, after three washes with DMEM, the monolayers were incubated in 2.5% glutaraldehyde–2% formaldehyde for 15 min, followed by 2.5% paraformaldehyde–2.5% glutaraldehyde–0.1% sodium cacodylate (pH 7.4), and then postfixed in 1% osmium tetroxide–0.1% sodium cacodylate. Cells were dehydrated in increasing concentrations of ethanol and embedded in epoxy resin (TAAB Laboratories Equipment, Ltd., United Kingdom). Ultrathin sections were stained with Reynolds lead citrate (23).

Vaccination and virus challenge of sheep vaccinated with DISC viruses. To assess the efficacy of the vaccine in sheep, groups of 6-month-old male and female healthy sheep (Crossbred Pre-Alpes) were used. The animals were free of respiratory, digestive, umbilical, parasitic and osteo-articular diseases and were seronegative against BTV and Q fever. For the first vaccine trial, three groups of six animals each were segregated at random. Group 1 was the control group, inoculated with lysate from VP6-complementing cell line. Groups 2 and 3 were inoculated with two different vaccine candidates, BTVE1 and BTVD2 (10^8 PFU/sheep). The samples consisted in crude cell lysate from VP6-complementing cell line infected with one of the VP6-deficient viruses. On day 0, the animals were vaccinated subcutaneously on the lateral side of the thorax, and on day 22, the animals received a second vaccination. On day 42, all animals were challenged with 1 ml of BTV-1 by means of several intradermal injections in the inner side of the right thigh. At the end of the experiment, all animals were sacrificed.

For testing the ability of the vaccine to elicit immune response with one or two doses, two groups of animals were inoculated with either 1×10^8 PFU/animal once (one-dose group) or 5×10^7 PFU/animal twice (two-dose group) 21 days apart. The efficacy of the reassortant BTV1/8D1 as an immunogen was tested by vaccination of a group of sheep with 10^8 PFU/ml using a prime and boost protocol. On day 42, all animals were challenged with an infectious BTV-8 virus. Both virus strains, BTV-1 and BTV-8, used for challenge in all trials are tissue culture adapted and induced a weak clinical disease in animals.

Sample collections from infected sheep. A classical clinical examination of all sheep was undertaken from day 1 to 7, from day 20 to 28, and from day 48 to 59. Blood sampling (by jugular puncture) in plain tubes was carried out to monitor the antibody response to BTV antigens on days 0, 11, 22, 31, 42, 49, 56, and prior

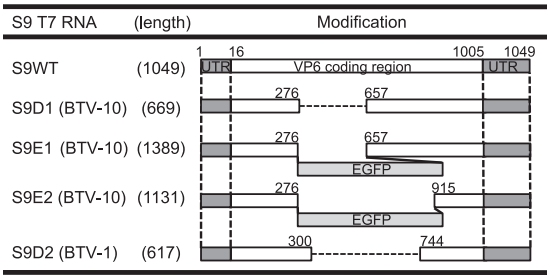

FIG. 1. Schematic representation of the changes introduced in the segment S9 of BTV. On the left, the name of each mutation is indicated. D1, E1, and E2 S9 segments were derived from BTV-10, while D2 was derived from BTV-1 as shown. Numbers indicate nucleotide positions in S9 where deletions were introduced. Insertion of the EGFP marker gene is also shown. The total length of each segment is indicated.

to euthanasia (day 66). The collected sera were heat inactivated for 30 min at 56°C. The sera were tested with a commercial enzyme-linked immunosorbent assay (ELISA) kit (ID-VET) with BTV VP7 antigen prebound to the plates according to the manufacturer's protocols. To test viremia in blood samples, whole blood was collected in EDTA tubes to enable the detection of the circulating virus. The viral RNA was extracted using a commercial extraction kit (Qiagen) and specific BTV RNA was quantified by quantitative reverse transcription-PCR (RT-PCR) using an Adiavet BTV detection kit (Adiagene, France) according to the protocols developed by Toussaint et al. (34).

Detection of neutralizing antibody response in the sera of vaccinated animals. The development of neutralizing antibody response to the BTV was assessed in BSR cells by serum neutralization with 50 50% tissue culture infective dose(s) ($TCID_{50}$) as previously described (16, 35). All dilutions were performed at least in duplicate and assayed independently twice. The neutralizing titer of each serum was defined as the highest dilution allowing neutralization of the 100 infectious particles.

RESULTS

Construction of VP6-deficient BTV mutants. In our previous report using the reverse genetics system, we demonstrated that a mutant BTV S9 segment, E1, bearing a major deletion in the coding region of VP6 (nucleotides [nt] 276 to 657) and a copy of the marker gene EGFP (Fig. 1) could be rescued and produce a viable virus when the VP6 protein was provided in *trans* using the complementary cell line BSR-VP6 (17). Furthermore, this mutant virus could not replicate in normal BSR cells, suggesting that BTV lacking functional VP6 could serve as a vaccine strain. To investigate this further, a series of truncated S9 RNA segments, which contain deletions in the coding region of VP6 of either BTV-1 or BTV-10, were derived (Fig. 1). The rationale for using S9 of BTV-1 and BTV-10 was to compare the genetic stability of these deletion mutant viruses in a complementary cell line that expresses BTV-10 VP6. Although the sequence of VP6 protein is highly conserved across all BTV serotypes and between BTV-1 and BTV-10, amino acid residues are 89% conserved, it is important to establish that BTV-10 VP6 complementary cells are compatible with other serotypes. The three mutations designed in S9 included either a deletion similar to the E1 (nt 276 to 657) but without any substitution of a marker gene (D1) or a major deletion (nt 276 to 915) but substituted with EGFP gene (E2); both D1 and E2 were derived from S9 of BTV-10, similar to E1. The third S9 deletion (nt 300 to 744) mutant (D2) was derived from BTV-1.

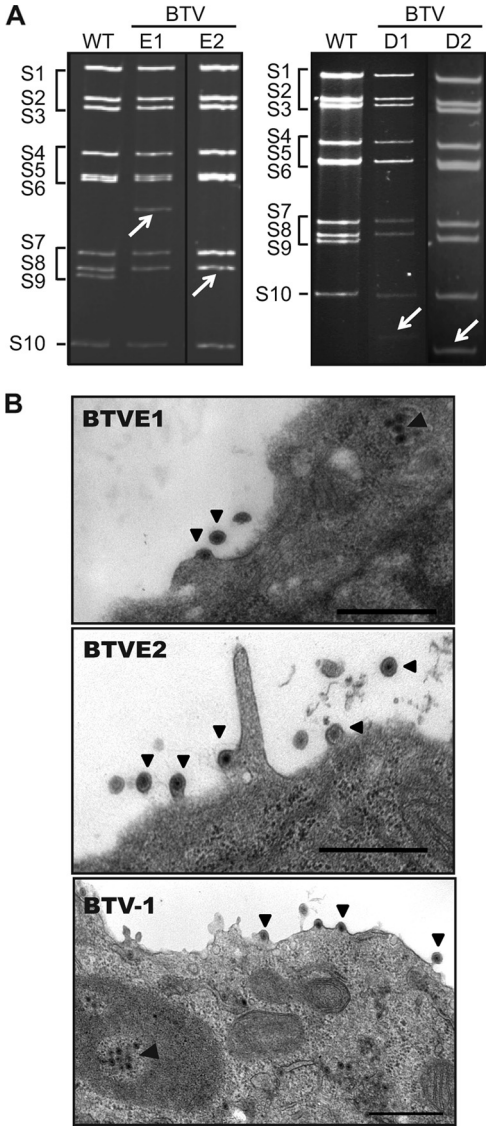

FIG. 2. Rescue of VP6-deficient viruses in complementing cell line. (A) Pattern of genomic dsRNA purified from infected cells with BTVE1, BTVE2, BTVD1, and BTVD2 was analyzed on a 9% nondenaturing polyacrylamide gel. As a control, genomic dsRNA from the WT virus was included. The position of the corresponding S9 in each VP6-defective virus is indicated by white arrows. (B) Structural analysis of ultrathin sections of complementing cells infected with BTVE1 (top panel) and BTVE2 (middle panel). Black arrowheads indicate particles. A cell section of normal BSR cells infected with BTV-1 was included as a control (bottom panel). Bar, 500 nm.

Each virus was recovered in the BSR-VP6 complementing cell line and the profile of the genomic dsRNAs of each mutant virus was analyzed by native-PAGE (Fig. 2A). The presence of the designed mutation in each case was confirmed by sequencing of the corresponding RT-PCR product (data not shown). No unexpected changes were detected, indicating that all mutant segments were functional in genome packaging and replication in complementary BSR-VP6 cells. Virus stocks were grown in BSR-VP6 complementing cells and kept at a low passage number (<5) for all further experiments.

To characterize the replication of the VP6-deficient viruses

in complementary BSR-VP6 cells, an ultrastructural analysis was performed. Cells were infected with BTVE1 or BTVE2, fixed at 24 h postinfection and processed for cell sectioning (Fig. 2B, top and middle panels). As a control, normal BSR cells were infected with BTV-1 wild type (WT) and processed similarly. Virus particles were detected throughout the cytoplasm and also being released from the plasma membrane of cells infected with the mutant viruses, similar to the WT virus infection (Fig. 2B, bottom panel), indicating that there is no major difference in virus replication between mutant viruses in the complementing cells and WT virus in normal cells.

VP6-deficient viruses could not replicate in normal cells but synthesize viral proteins. The fundamental characteristic of a DISC virus is that, while it may complete a replication cycle in a complementing cell line, it is unable to do so in normal cells. To assess the replication capability of the VP6 mutant viruses in normal BSR cells, the total infectious virus titer (cell-associated and cell-free virus) was calculated at intervals and compared to WT BTV. Briefly, BSR cells were infected at an MOI of 0.01 with each of VP6-deficient viruses D1, E1, and E2 and harvested at 2, 24, 48, and 96 h postinfection as described in Materials and Methods. As shown in Fig. 3A, the titers of all VP6-deficient viruses decreased throughout the time course, whereas the titer of WT BTV-1 increased. Although the VP6-deficient viruses showed no replication in normal cells, BTV proteins synthesized by incoming viruses should be detectable (17). Thus, normal BSR cells were infected with BTVE1, BTVD1, and BTVE2 viruses at an MOI of 5, and the expression of BTV proteins was detected by Western blotting. Both structural (VP1, VP4, VP5, and VP7) and nonstructural (NS1, NS2, and NS3/NS3A) proteins were detected in lysates from BSR cells infected with mutant viruses, and there were no major differences detected in comparison to the lysate from WT virus-infected cells (Fig. 3B).

To compare these three mutant viruses with the defective BTVD2 virus that possess the S9 derived from BTV-1, its growth curve in normal BSR cells and its protein expression profile were assessed (Fig. 3C). Similar to the mutants BTVE1, -D1, and -E2, no virus replication at 72 h postinfection in normal cells was detected (Fig. 3C, left panel). Despite no replication, viral proteins could be detected in BSR cells infected with BTVD2. Synthesis of the major viral NS2 protein is shown (Fig. 3C, right panel). These results showed that each VP6-deficient mutant virus is replication defective in normal BSR cells despite the parental serotype and variation in their open reading frame regions.

Moreover, sections of normal BSR cells infected with VP6-defective viruses showed no virion particles within the cells or at the plasma membrane (data not shown), indicating that the normal virus assembly and maturation were perturbed. These data demonstrated that the VP6 mutant viruses have the characteristic features of DISC viruses in lacking the ability to complete a replication cycle in normal cells or produce infectious virions but, importantly for a candidate vaccine strain, the synthesis of viral proteins still occurs.

BTV-1 DISC virus strains elicit neutralizing immune responses in sheep that confer complete protection against virulent virus challenge. The expression of viral proteins without replication in normal BSR cells encouraged us to test whether DISC virus strains could be used as safe vaccines in animals.

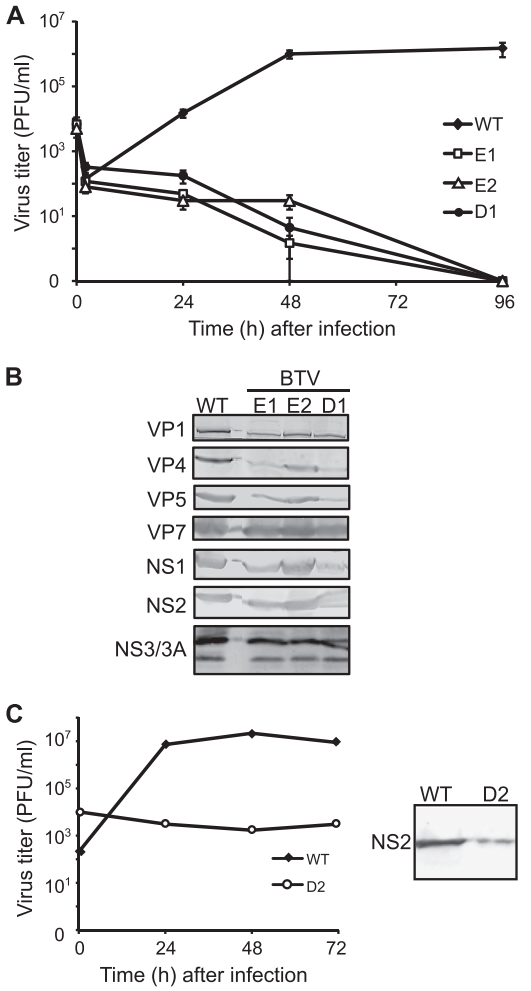

FIG. 3. Characterization of VP6-deficient viruses in normal BSR cells. (A) Virus growth kinetics of VP6-deficient viruses BTVE1, BTVE2, and BTVD1. Total virus titer was determined by plaque assay at 0, 2, 24, 48, and 96 h postinfection and plotted as PFU/ml in logarithmic scale. As a control, cells were infected with WT BTV. (B) Protein expression pattern in BSR cells infected with the VP6-deficient viruses BTVE1, BTVE2, and BTVD1. BSR cells were infected with viruses at an MOI of 5 and analyzed 48 h postinfection by Western blotting with specific antibodies against structural (VP1, VP4, VP5, and VP7) and nonstructural (NS1, NS2, and NS3/NS3A) proteins. Cell lysate from BSR infected with WT BTV-1 was included as a control. (C) Virus growth (PFU/ml) and protein expression (NS2) in BSR cells infected with BTVD2 are shown.

Thus, as a proof of concept, only two DISC viruses, BTVE1 and BTVD2, were used to inoculate two groups of six sheep, each with 10^8 PFU/sheep in a first set of experiments. As a control, a group of animals was inoculated with VP6-complementing cell lysate. All animals were boosted with the same virus strains or VP6-cell lysate at 21 days after the first vaccination, and serum samples were collected at regular intervals to monitor the replication capacity and the immune response to VP6-deficient viruses. All animals in vaccinated groups seroconverted (positive threshold $> 35\%$) by 7 days postvaccination when assessed by a group-specific VP7 competitive ELISA, in contrast to the control group where specific anti-BTV antibodies were not detected (Fig. 4A). Furthermore, the

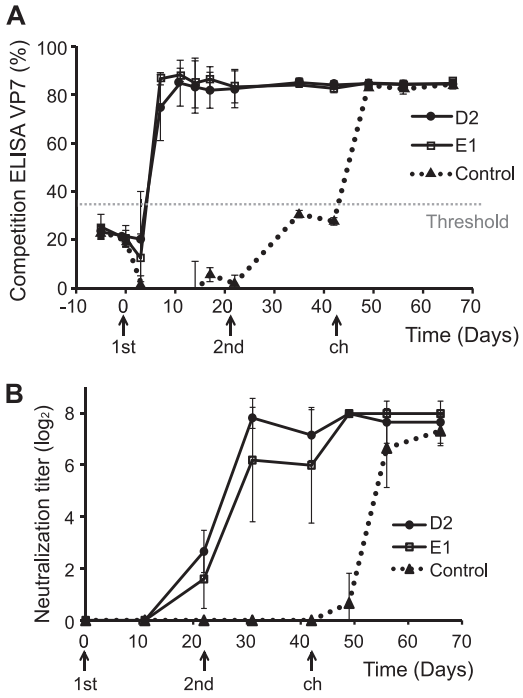

FIG. 4. Immune response of animals vaccinated with the VP6-defective viruses BTVE1 and BTVD2. (A) Seroconversion of vaccinated sheep using competitive ELISA specific to BTV VP7 antigen. "1st" and "2nd" indicate the first vaccination and boost, respectively. "Ch" indicates the challenge with virulent BTV-1 strain. A 35% competition threshold is indicated. (B) Virus neutralization titers in serum samples of vaccinated animals were determined by $TCID_{50}$ as described in Materials and Methods. The titers are shown in a $\log_2$ scale.

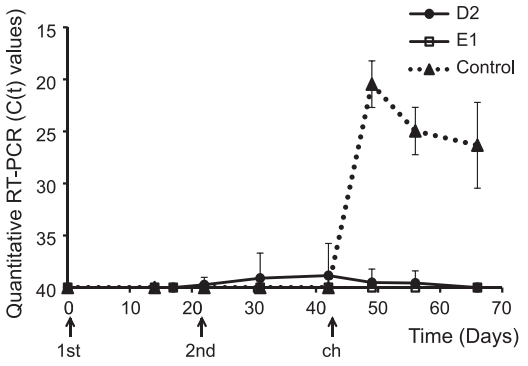

FIG. 5. Viremia in animals vaccinated with BTVE1 and BTVD2. BTV genomic RNA was detected in blood samples by quantitative real-time RT-PCR at different intervals in the vaccine schedule. Mean cycle thresholds (C_t) and standard deviations are shown considering negative results as $C_t \geq 40$.

neutralizing antibody titers of all animal sera were determined by serum neutralization assay (Fig. 4B). All animals immunized with the disabled virus strains produced a neutralizing response, whereas serum from the control animal group did not neutralize infectivity. These results suggest that the two disabled vaccine candidates were able to raise BTV antibody responses, including a neutralizing response in the vaccinated animals.

Whether the elicited antibodies were sufficient to protect the vaccinated sheep against virulent virus challenge was subsequently assessed. At 42 days postinoculation, all sheep were challenged with virulent BTV-1, and blood samples were taken at 49, 56, and 66 days. All samples were tested for both ELISA using VP7, as described above, and also assessed for neutralizing antibody titers. As shown in Fig. 4A, the control group had the VP7-antibody response in the ELISA only after challenge with infectious WT virus on day 42 onward, whereas the antibody response in vaccinated animals remained stable and over the positive threshold until the end of the experiment.

Similarly, the neutralizing antibody titers of the vaccinated animals after challenge (day 42) did not show any changes (Fig. 4B). In contrast, infection of the control animals elicited antibodies only after the challenge, indicating the infectivity of the challenge virus.

The DISC vaccine strains are unable to replicate in sheep but prevent challenge virus replication. The capacity of a BTV vaccine candidate to inhibit virus replication and viremia is critical for limiting the transmission of the virus by the insect vector. To test whether the DISC virus strains were able to replicate in sheep, a BTV-specific quantitative real-time RT-PCR test was performed at intervals (days 0 to 42) throughout the vaccination trial studies. Blood samples from each animal were used to detect BTV replication. No BTV genomic RNA was detected in blood samples from vaccinated animals (Fig. 5) with cycle threshold (C_t) values similar to the values determined for the control group between days 0 to 42, indicating that the vaccine viruses were not able to replicate in sheep. Only one animal presented a slightly lower C_t values after the boost (from 21 days onward), indicating that some BTV RNA was detected in this sheep. To determine whether this was due to a low level of replication of the DISC virus, a blood sample from this animal was used to inoculate embryonated eggs in order to detect replicating virus. No virus growth was observed in the inoculated eggs, suggesting that the lower C_t values detected in this particular animal were due to the detection of viral RNA derived from the vaccine virus (data not shown).

To evaluate virus replication in vaccinated animals after challenge with a virulent strain at day 42 postinoculation, blood samples were taken at 49, 56, and 66 days. As expected, no virus replication was detected in the vaccinated groups after challenge using this sensitive assay. The control group on the other hand, showed a clear reduction in the C_t values from 7 days postchallenge only (day 49), indicating virus replication, followed by a progressive reduction of the virus from blood circulation, as expected for BTV infection in sheep (Fig. 5). These results clearly indicate that in vaccinated animals the replication of virulent BTV was inhibited; thus, sheep were fully protected against an infectious virus challenge.

BTV-1 DISC virus confer protection from virus replication after one dose. Current vaccines are designed to be delivered as a prime-boost protocol, with at least two doses of the antigen delivered to each animal, but a single vaccination approach in which one dose could be used to provide sufficient protection from disease would be more desirable since it would be a less time-consuming and more cost-effective vaccine.

In addition, the vaccine is required to induce a protective level of antibody with the minimum dose. We investigated whether the DISC virus strains were able to induce antibodies

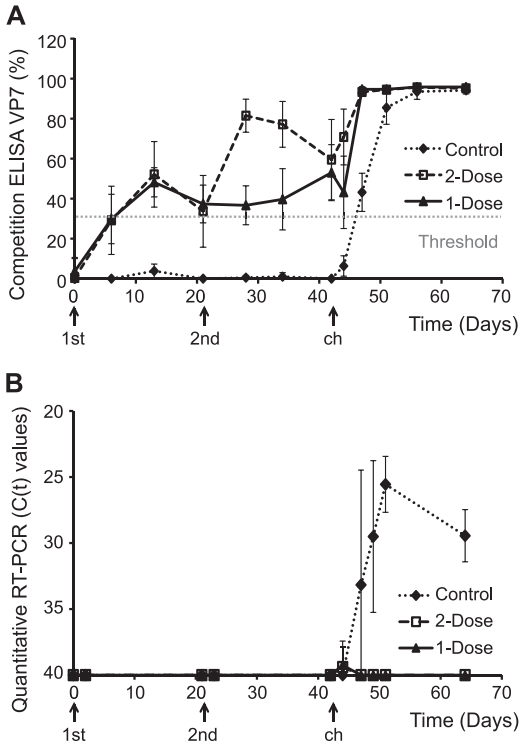

FIG. 6. Immune response of animals vaccinated with BTVE2 in a one- or two-dose scheme. Groups of sheep were vaccinated once with VP6-deficient virus at 1×10^8 PFU/sheep or twice at 5×10^7 PFU/sheep before challenge. (A) Seroconversion in both groups determined by competitive BTV VP7 ELISA. (B) Viremia in vaccinated and control animals determined by specific quantitative real-time RT-PCR.

against BTV antigens and prevent viremia with a single dose of 1×10^8 PFU/sheep and compared to a lower dose, 5×10^7 PFU/sheep, but with the same prime-boost regime as described above.

In this experiment, we used a virus strain (BTVE2) with a larger deletion in the VP6 gene (i.e., deletion of nt 276 to 915) also containing the EGFP marker gene. Two groups of six sheep were then inoculated with the BTVE2 virus at day 0, but the boost was administered to only one group at day 21 (two-dose group). On day 42, all animals were challenged with virulent BTV-1 virus.

Blood samples were collected from each animal from days 0 to 64, and each sample was assessed to determine the presence of BTV antibodies and the viral loads as described above. The seroconversion of all vaccinated animals was evident before the challenge (day 42) with 59% for the two-dose group and with 53% of the VP7-ELISA competition for the one-dose group (Fig. 6A). On day 44 (2 days after challenge), the antibody titer was still very high for the two-dose group and was slightly reduced for the one-dose group (Fig. 6A).

In both groups, animals elicited a neutralizing antibody response. Even though in the two-dose group, the animals were inoculated with only half of the virus amount (5×10^7 PFU/sheep) compared to that of the previous vaccination trial (10^8 PFU/sheep); nevertheless, all elicited neutralizing antibodies. However, in this trial the maximum neutralizing antibody titers, prior to the challenge (at 42 days) was only 16, much lower than the response detected in the first trial where the titer was up to 64.

Significantly, the serum neutralization assay showed that animals of the one-dose group had titers similar to the titers detected for animals in the two-dose group, suggesting that a single dose could elicit a neutralizing response in vaccinated animals. Three of the six animals vaccinated only once with 10^8 PFU/sheep had neutralizing antibody titers of approximately 4 to 16.

Although the neutralization titers in both one- and two-dose groups were lower compared to the first trials, the replication of virulent virus was completely abrogated in both groups as in the previous trial. There was no replication of the challenge virus in the vaccinated groups, even in one-dose group (Fig. 6B). However, virus replication was quite evident in the control group, when challenged with infectious BTV-1, and blood samples of these animals showed a clear reduction in the C_t values from 5 days (day 47) postchallenge (Fig. 6B). These results strongly suggest that all vaccinated sheep were protected even though they did not have high levels of neutralizing antibodies, including the sheep that received only a single dose of vaccination.

Characterization of the reassortant BTV1/BTV8 DISC virus strain. Since the core proteins are highly conserved across different BTV serotypes and gene segments readily reassort between different serotypes, we wanted to determine whether it is possible to generate DISC virus strains for another serotype using the same core composition of the BTV-1 DISC virus strain. The determinant of the serotype for BTV is the outermost capsid protein VP2 encoded by the segment S2. This segment S2 usually segregates with the corresponding segment 5 encoding the VP5 protein from the same serotype. Thus, to generate a defective reassortant virus, we replaced genome segments 2 and 5 with those from BTV-8 strain. Since the virus BTVD1 showed complete clearance from infected BSR cells (Fig. 3A), we decided to use the same deleted segment S9D1. This BTV1/BTV-8 (BTV1/8D1) virus was rescued and amplified in the complementing BSR-VP6 cell line as described above.

The dsRNA profile of the newly generated reassortant BTV1/8D1 clearly showed that the migration of the segment 5 (S5) was similar to the parental BTV8 and the faster migration for the segment 9 (S9) indicates that it had the designed deletion (Fig. 7A). Since no difference in the migration rate of segment 2 from parental BTV-1 and BTV-8 strains is evident, the identity of segment 2 in the reassortant virus was confirmed by partial sequencing of the RT-PCR product (data not shown).

The expression of viral proteins in normal or complementing VP6 cells infected with BTV1/8D1 was also tested. The major core structural (VP7) and nonstructural (NS2) proteins (derived from BTV-1) in both cell lines were detected, and there were no detectable differences from the parental viruses in the two cell lines (Fig. 7B). Although the protein expression was similar to the parental viruses, no replication of the reassortant virus was detected in normal BSR cells (Fig. 7C), similar to the results obtained with the parental mutant BTVD1 (compare with Fig. 3A). These results suggested that the reassortant virus could not replicate in normal BSR, but virus proteins can

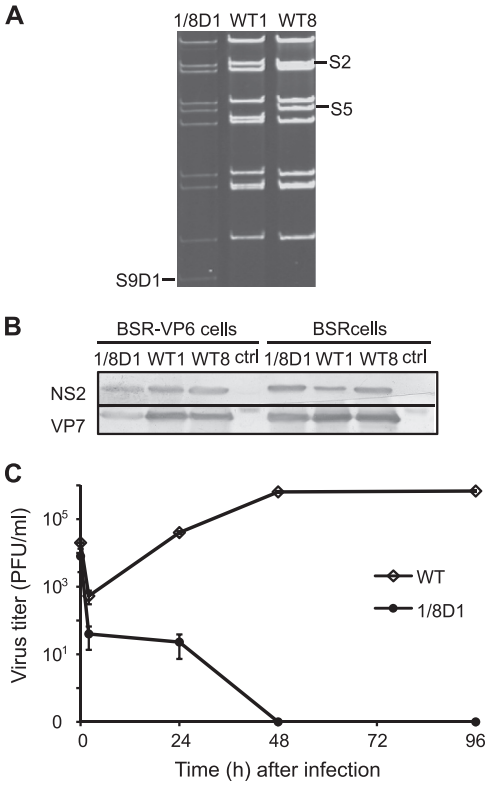

FIG. 7. Characterization of reassortant BTV1/BTV8 VP6-defective virus. Mutant virus BTV1/8D1 was rescued in complementing VP6 cell line. (A) Genomic dsRNA was purified from infected cells and analyzed by nondenaturing-PAGE. (B) Structural (VP7) and nonstructural (NS2) proteins were detected by Western blotting in normal or complementing VP6 cells. Cells were infected with BTV-1 (WT1), BTV-8 (WT8), or BTV1/8D1 (1/8D1). Mock-infected VP6 complementing and normal BSR cells were included as negative controls (ctrl). (C) Virus growth curve in normal BSR cells infected with BTV1/8D1 determined by plaque assay and calculated as PFU/ml.

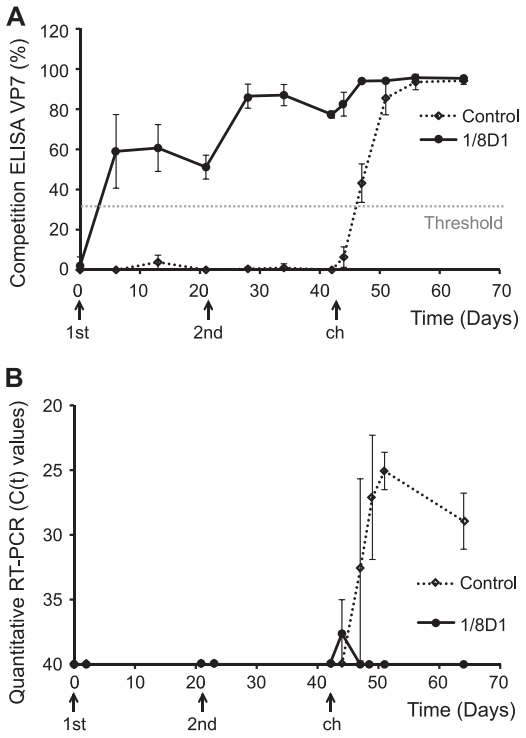

FIG. 8. Immune response of animals vaccinated with BTV1/8D1. (A) Seroconversion of animals vaccinated with BTV1/8D1 determined by competition ELISA. (B) Viremia in animals vaccinated with the reassortant BTV1/8D1 and challenged with a virulent BTV-8 strain.

still be expressed, indicating that its behavior is similar to the BTV-1 VP6 mutants.

The reassortant BTV1/8D1 is able to trigger antibody responses in sheep and prevent viremia upon virulent virus challenge. The BTV1/8D1 DISC virus was used for immunization of a group of six sheep with a prime and boost regime similar to that described above using 10^8 PFU/sheep. As with BTV1 DISC virus strain immunization, all immunized animals seroconverted prior to the boost, and this response was even stronger after the second vaccination (Fig. 8A). Serum neutralization responses of all animals to BTV-8 were assessed with the sera collected at intervals throughout the trial. At 42 days after the first inoculation, one sheep presented the maximum titer of 128, three sheep had neutralization antibody titers of 64, and one had a titer of 32. The lowest neutralization antibody titer was 16 for only one sheep. These results indicated that the reassortant virus based on the BTV-1 genetic background elicited a neutralizing antibody response against the highly pathogenic BTV-8.

The viremia in vaccinated animals was also analyzed by real-time RT-PCR as described above. After challenge, there was a transient detection of viral RNAs in vaccinated animals near the detection limit of the assay, which is consistent with the detection of the DISC virus genomic RNAs (Fig. 8B). However, there was no replication of the challenge virus in any of the vaccinated sheep, including the animal with the lowest neutralization antibody titer.

These results demonstrate that the reassortant virus containing the outer capsid proteins from an alternate serotype can raise a neutralization response and prevent viremia after challenge with the second serotype, in vaccinated sheep. Together, these data strongly support that the designed replication deficient virus strains are highly efficacious vaccine candidates, and the reverse genetics systems for BTV could provide a rapid method to generate vaccines for emerging serotypes.

DISCUSSION

A number of different strategies for obtaining a vaccine to confer protection against BTV have been developed in the past, including the two vaccine types available in the market: inactivated and attenuated live virus vaccines. However, problems associated with these two vaccines, such as incomplete attenuation that can produce enough viremia for transmission, potential reversion to virulence, and reassortment with a field strain makes essential the development of new vaccines (11, 20, 29). Inactivated vaccines have the associated disadvantages of needing larger doses to raise an immune response and the requirement for every batch to be tested to ensure complete inactivation of infectivity. Thus, a variety of alternate approaches have been undertaken to generate BTV vaccines,

many of which have been tested in laboratory conditions with some level of success (1, 13, 14, 21, 30, 32). In fact, our laboratory has demonstrated strong evidence supporting the VLPs as a good alternative to current vaccines (9, 28, 30, 32, 33). A completely different strategy, such as defective viruses which could trigger protective immune responses but do not replicate in vaccinated animals, has not been possible to develop due to the lack of a reverse genetics system for BTV in the past. The advantages of such vaccines are that these vaccines would be as safe as an inactivated vaccine but preserve the expression of viral proteins at the natural sites of infection, as with live vaccines. This would be safer than current live virus vaccine strains with respect to the risk of reversion to virulence. In fact, a virus that cannot replicate in animals is less likely to revert or reassort with a field strain than the typical attenuated vaccines. However, this assumption should be tested with a larger number of animals.

An appropriate reverse genetics system has now been established in our laboratory. In this report we present evidence of the protection against challenge with infectious virus in sheep vaccinated with different defective viruses recovered using this system.

The viral core protein VP6, encoded by RNA segment 9 (S9), is an essential enzymatic protein for virus replication, and thus it is a suitable target for generating a replication-deficient virus strain (17). In the present study, we generated four different VP6-defective virus strains using reverse genetics systems in combination with a complementary cell line expressing a functional VP6 protein, which has been developed previously (17). Each rescued virus was characterized and compared to a VP6-deficient virus that has been successfully rescued and characterized previously (17). These constructs included mutations in segments S9 from two different BTV serotypes. It was important to establish that the deficiency in virus replication was intrinsic to the deletion and/or modification in the segment and was not related to the origin of the S9. No obvious differences in terms of replication in normal cells between viruses containing S9 either from BTV-1 or from BTV-10 were detected, demonstrating that the deficiency in the replication in these viruses was due to the mutation itself. Although no virus replication was detected in normal cells infected with the VP6-deficient viruses, several viral proteins were detected within these cells, similar to that of a WT virus infection.

When groups of sheep were immunized with these VP6-defective DISC viruses, it was evident that each tested animal produced an immune response soon after the first vaccination. Some differences in the ELISA titers among all trials were detected, probably due to the differences in the deficient viruses used as vaccines. Further, a neutralizing antibody response was detected in the sera of all animals vaccinated with two doses of either 1×10^8 or 5×10^7 PFU/sheep that correlated with protection against viremia (real-time RT-PCR) in challenged sheep. More importantly, the group of sheep with only one dose of the immunization showed levels of specific VP7 antibodies at 42 days postimmunization similar to the two-dose group, suggesting that one dose is sufficient to elicit virus-specific antibody response and maintain it at least for 42 days. Further, a single immunization could prevent the replication of a challenged virus, indicating that protection against challenge was provided by only one-dose of vaccination. These results have the potential for commercial exploitation since a vaccine requiring a single dose will be less labor-intensive and more cost-effective than the current BTV vaccines that require two doses.

Complex aspects for the development of a vaccine against BTV is that there are 24 serotypes and that the vaccines are serotype specific. Although there is some cross-reaction, animals vaccinated against one serotype will not be protected against infection with a different serotype. It is essential to have a system that allows the development of vaccines to different emerging serotypes rapidly.

We presented strong evidence here that it is possible to generate a vaccine virus for different serotypes rapidly by using the reverse genetics system. Our data demonstrated that the genetic background of one serotype (BTV-1) can be used to generate defective virus of another serotype by replacing the genome segments encoding the variable outer capsid proteins VP2 and VP5 with the serotype of a highly virulent virus (BTV-8). Moreover, this defective reassortant virus was able to elicit a specific neutralizing antibody response against BTV-8 in sheep and conferred full protection from a challenge with a virulent strain of this serotype. The construction of a BTV-8 DISC strain using the backbone of BTV-1 DISC virus strain provides proof of principle for the generation of similar vaccines for other BTV serotypes as required. Such DISC vaccine candidates would expand the scope for BTV vaccines considerably, since both attenuated and killed vaccines that are currently used have a history of safety issue.

The DISC virus strains are also compliant with an essential aspect for BTV control, since vaccinated animals could be easily distinguishable from the infected animals (DIVA). The absence of antibodies against VP6 can be used as a marker for vaccinated and noninfected animals. Further, we also designed viruses to carry a marker gene (EGFP) in order to analyze whether this marker can be also used as a second indicator for vaccinated animals. We predict that animals infected with this EGFP/DISC virus would raise antibodies not only against BTV proteins but also against the marker protein and antibodies, present only in vaccinated animals, which could easily be detected. However, further studies will be needed to confirm this issue.

An additional advantage of the EGFP/DISC virus strain is that that it could be used for the detection of neutralizing antibodies in infected serum samples based on marker detection assay. In this case, the neutralizing response can be determined by quantification of EGFP signal by incubating the mutant virus with the serum to be tested. A similar approach has been used for detection of antibodies against influenza virus using a mutant influenza virus that carries a copy of GFP marker (24).

Overall, the data presented in this report demonstrate that the BTV DISC virus strains are potentially suitable vaccine candidates with many desirable criteria over the current BTV vaccines. We also emphasize here the power of the reverse genetics system for the rapid generation of serotype-specific orbivirus vaccines.

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REFERENCES

1. **Boone, J. D., et al.** 2007. Recombinant canarypox virus vaccine coexpressing genes encoding the VP2 and VP5 outer capsid proteins of bluetongue virus induces high level protection in sheep. Vaccine **25:**672–678.
2. **Boyce, M., C. C. Celma, and P. Roy.** 2008. Development of reverse genetics systems for bluetongue virus: recovery of infectious virus from synthetic RNA transcripts. J. Virol. **82:**8339–8348.
3. **Boyce, M., and P. Roy.** 2007. Recovery of infectious bluetongue virus from RNA. J. Virol. **81:**2179–2186.
4. **Boyce, M., J. Wehrfritz, R. Noad, and P. Roy.** 2004. Purified recombinant bluetongue virus VP1 exhibits RNA replicase activity. J. Virol. **78:**3994–4002.
5. **Castrucci, M. R., P. Bilsel, and Y. Kawaoka.** 1992. Attenuation of influenza A virus by insertion of a foreign epitope into the neuraminidase. J. Virol. **66:**4647–4653.
6. **Celma, C. C., and P. Roy.** 2009. A viral nonstructural protein regulates bluetongue virus trafficking and release. J. Virol. **83:**6806–6816.
7. **Elbers, A. R., et al.** 2008. Field observations during the Bluetongue serotype 8 epidemic in 2006. II. Morbidity and mortality rate, case fatality and clinical recovery in sheep and cattle in the Netherlands. Prev. Vet. Med. **87:**31–40.
8. **Forzan, M., M. Marsh, and P. Roy.** 2007. Bluetongue virus entry into cells. J. Virol. **81:**4819–4827.
9. **French, T. J., J. J. A. Marshall, and P. Roy.** 1990. Assembly of double-shelled, viruslike particles of bluetongue virus by the simultaneous expression of four structure proteins. J. Virol. **64:**5695–5700.
10. **Gloster, J., P. S. Mellor, A. J. Manning, H. N. Webster, and M. C. Hort.** 2007. Assessing the risk of windborne spread of bluetongue in the 2006 outbreak of disease in northern Europe. Vet. Rec. **160:**54–56.
11. **Gonzalez, J. M., et al.** 2010. Possible adverse reactions in sheep after vaccination with inactivated BTV vaccines. Vet. Rec. **166:**757–758.
12. **Halfmann, P., et al.** 2009. Replication-deficient Ebolavirus as a vaccine candidate. J. Virol. **83:**3810–3815.
13. **Huismans, H., N. T. van der Walt, M. Cloete, and B. J. Erasmus.** 1987. Isolation of a capsid protein of bluetongue virus that induces a protective immune response in sheep. Virology **157:**172–179.
14. **Inumaru, S., and P. Roy.** 1987. Production and characterization of the neutralization antigen VP2 of bluetongue virus serotype 10 using a baculovirus expression vector. Virology **157:**472–479.
15. **Maan, S., N. S. Maan, N. Ross-smith, et al.** 2008. Sequence analysis of bluetongue virus serotype 8 from the Netherlands 2006 and comparison to other European strains. Virology **377:**308–318.
16. **Marshall, J. J. A., and P. Roy.** 1990. High level expression of the two outer capsid proteins of bluetongue virus serotype 10: their relationship with the neutralization of virus infection. Virus Res. **15:**189–195.
17. **Matsuo, E., and P. Roy.** 2009. Bluetongue virus VP6 acts early in the replication cycle and can form the basis of chimeric virus formation. J. Virol. **83:**8842–8848.
18. **Mertens, P. P., J. N. Burroughs, and J. Anderson.** 1987. Purification and properties of virus particles, infectious subviral particles, and cores of bluetongue virus serotypes 1 and 4. Virology **157:**375–386.
19. **Mintiens, K., et al.** 2008. Possible routes of introduction of bluetongue virus serotype 8 into the epicentre of the 2006 epidemic in northwestern Europe. Prev. Vet. Med. **87:**131–144.
20. **Osburn, B. I.** 1994. The impact of bluetongue virus on reproduction. Comp. Immunol. Microbiol. Infect. Dis. **17:**189–196.
21. **Perrin, A., et al.** 2007. Recombinant capripoxviruses expressing proteins of bluetongue virus: evaluation of immune responses and protection in small ruminants. Vaccine **25:**6774–6783.
22. **Purse, B. V., et al.** 2005. Climate change and the recent emergence of bluetongue in Europe. Nat. Rev. Microbiol. **3:**171–181.
23. **Reynolds, E. S.** 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol. **17:**208–212.
24. **Rimmelzwaan, G. F., et al.** 2011. Use of GFP-expressing influenza viruses for the detection of influenza virus A/H5N1 neutralizing antibodies. Vaccine **29:**3424–3430.
25. **Roy, P.** 2008. Functional mapping of bluetongue virus proteins and their interactions with host proteins during virus replication. Cell Biochem. Biophys. **50:**143–157.
26. **Roy, P.** 1996. Orbivirus structure and assembly. Virology **216:**1–11.
27. **Roy, P.** 2007. Orbiviruses and their replication, p. 1975–1997. *In* D. M. Knipe and P. M. Howley (ed.), Fields virology, 5th ed., vol. 2. Lippincott/The Williams & Wilkins Co., Philadelphia, PA.
28. **Roy, P., D. H. Bishop, H. LeBlois, and B. J. Erasmus.** 1994. Long-lasting protection of sheep against bluetongue challenge after vaccination with virus-like particles: evidence for homologous and partial heterologous protection. Vaccine **12:**805–811.
29. **Roy, P., M. Boyce, and R. Noad.** 2009. Prospects for improved bluetongue vaccines. Nat. Rev. Microbiol. **7:**120–128.
30. **Roy, P., T. French, and B. J. Erasmus.** 1992. Protective efficacy of virus-like particles for bluetongue disease. Vaccine **10:**28–32.
31. **Roy, P., J. J. Marshall, and T. J. French.** 1990. Structure of the bluetongue virus genome and its encoded proteins. Curr. Top. Microbiol. Immunol. **162:**43–87.
32. **Roy, P., T. Urakawa, A. A. Van Dijk, and B. J. Erasmus.** 1990. Recombinant virus vaccine for bluetongue disease in sheep. J. Virol. **64:**1998–2003.
33. **Stewart, M., et al.** 2010. Validation of a novel approach for the rapid production of immunogenic virus-like particles for bluetongue virus. Vaccine **28:**3047–3054.
34. **Toussaint, J. F., C. Sailleau, E. Breard, S. Zientara, and K. De Clercq.** 2007. Bluetongue virus detection by two real-time RT-qPCRs targeting two different genomic segments. J. Virol. Methods **140:**115–123.
35. **van der Walt, N. T.** 1980. A haemagglutination and haemagglutination inhibition test for bluetongue virus. Onderstepoort J. Vet. Res. **47:**113–117.
36. **Van Dijk, A. A., and H. Huismans.** 1988. In vitro transcription and translation of bluetongue virus mRNA. J. Gen. Virol. **69**(Pt. 3)**:**573–581.
37. **Verwoerd, D. W.** 1969. Purification and characterization of bluetongue virus. Virology **38:**203–212.
38. **Weiner, M. P., et al.** 1994. Site-directed mutagenesis of double-stranded DNA by the PCR. Gene **151:**119–123.