NOTES

Interference of Vaccination against Bluetongue Virus Serotypes 1 and 8 with Serological Diagnosis of Small-Ruminant Lentivirus Infection

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The effects of the recent vaccinations against bluetongue virus serotype 1 (BTV-1) and BTV-8 in Europe on the reliability of enzyme-linked immunosorbent assays (ELISAs) currently used for diagnosis of small-ruminant lentivirus (SRLV) infection were examined. Primary vaccination against BTV-8 in goats induced an increase in reactivity that did not exceed 3 months in a whole-virus indirect ELISA and a competitive ELISA based on the gp135 glycoprotein. Subsequent BTV-1/8 vaccination extended the time scale of false-positive reactivity for up to 6 months. These results are of relevance for SRLV-monitoring programs.

Maedi-visna virus (MVV) and caprine arthritis-encephalitis virus (CAEV) are two small-ruminant lentiviruses (SRLVs) which cause inflammatory and debilitating diseases in sheep and goats (10). SRLV infections are widespread throughout the world, and the inherent economic losses have led to the establishment of control and eradication programs for both goats and sheep in several countries (1, 5, 7, 11, 12, 13, 14, 15, 17). Due to the lack of medical treatment and vaccines, these programs have relied upon periodic serological testing, the slaughter of seropositive animals, and the introduction of animals only from certified SRLV-free flocks. Serological testing of SRLV infections is generally performed by enzyme-linked immunosorbent assay (ELISA) in routine diagnosis and by Western blotting (WB) or agar gel immunodiffusion (AGID) in confirmatory tests. Currently available ELISAs include either indirect ELISAs based on whole virus, recombinant proteins, and/or synthetic peptides as antigens or competitive ELISAs using antiviral monoclonal antibodies (2). These tests are based on either CAEV-like or MVV-like strains but are usually employed for the detection of infection in both sheep and goats, since the two SRLV genotypes share cross-reacting epitopes in all structural proteins (4).

In France, SRLV-monitoring programs were implemented on a voluntary basis in the early 1990s at the national level. As a result of a comparative study carried out at the French reference laboratory for SRLV, the Chekit CAEV/MVV ELISA from Bommeli AG/Idexx Laboratories and the LSIIVET CAEV/MVV blocking ELISA (L-ELISA) from VMRD/LSI Laboratories were selected for application in routine diagnosis, and positive sera were analyzed by WB as a confirmatory test. The C-ELISA is a whole-virus indirect assay, and the L-ELISA is a competitive assay based on the gp135 envelope protein. In 2008, many MVV-free flocks tested positive by ELISA despite a good adherence to the program’s guidelines. Similarly, new ELISA-positive results were obtained from rams in artificial insemination centers, although these animals originated from MVV-free flocks and had been seronegative for several years. The emergence of new cases of ELISA positivity was also reported but to a lesser extent in goat herds that had been CAEV free for many years. However, positive ELISA responses for most of the SRLV-free flocks were rarely confirmed by WB, suggesting the occurrence of a drawback in routine diagnosis of SRLV infection in sheep and goats.

Bluetongue is an arthropod-borne, noncontagious disease of domestic and wild ruminants, particularly affecting sheep with severe clinical disease (8). The etiologic agent, namely, bluetongue virus (BTV), belongs to the genus Orbivirus of the family Reoviridae. Recent outbreaks due to BTV serotype 8 (BTV-8) and BTV-1 in the north and southwest of France, respectively (9, 18), led the French veterinary authorities to implement a compulsory campaign of BTV vaccination in livestock since 2008. Surprisingly, epidemiological data revealed that the number of false-positive ELISA responses in SRLV-free flocks seemed to be related to the delay between BTV vaccination and serum collection for SRLV serological testing, suggesting a possible interference of BTV vaccination in routine diagnosis of SRLV infection. To address this hypothesis, a retrospective analysis was first performed on a collection of blood samples collected bimonthly from 23 BTV-8-vaccinated goats which were kept in the experimental station at the French reference laboratory for SRLV. The BTV-8 vaccination was performed in August 2008 and included two subcutaneous injections performed at 4-week intervals, according to the manufacturer’s instructions (Btvpur AlSap 8; Mérial). The statuses of animals for SRLV infection prior to the BTV-8 vaccination were determined by ELISA, WB, and PCR. Twenty-
one goats were negative in all tests, and two goats were positive.

The ELISA reactivity levels of plasma samples obtained from the 21 uninfected goats following the primary vaccination against BTV-8 are shown in Fig. 1 and Table 1. All samples showed increased reactivity in the C-ELISA at day 47 postvaccination, with 13 of them (62%) testing positive, according to the cutoff value of the assay. Subsequently, all samples tested negative at day 101 postvaccination, and reactivity levels stably settled into the negative range over a period of 6 months. Analysis of plasma samples using the L-ELISA revealed no positive responses during the same period of investigation. However, the reactivity profile obtained was similar to that obtained using the C-ELISA, with a peak of reactivity at day 47 postvaccination. Samples from the two CAEV-infected goats consistently tested positive in both of the ELISAs (data not shown).

During the last serological testing of SRLV-free flocks, false-positive responses in both the C-ELISA and L-ELISAs were obtained using sera collected more than 3 months after BTV vaccination. These findings differed from our results showing that primary vaccination against BTV-8 induced positive responses that did not exceed 3 months in the C-ELISA and a peak of reactivity that did not reach the positive threshold in the L-ELISA. An anamnestic immune response induced by successive vaccinations against BTV-8 and cumulative effects due to dual vaccinations against BTV-8 and BTV-1 could be explanations for the increased time scale of false-positive reactivity under field conditions. For elucidation of this hypothesis, all goats investigated in this study were subjected to a second BTV vaccination 11 months (day 326) following the first one. Animals received three subcutaneous injections of inactivated monovalent vaccines (BTV-8, BTV-8/1, and BTV-1) at 4-week intervals, according to the manufacturer's instructions (Btvpur AlSap 8, obtained from Mérial, and Syvazul 1, obtained from Virbac). Blood samples were collected on the day of vaccination and every 10 days over a period of up to 6 months postvaccination. As shown in Fig. 2 and Table 1, sera from uninfected goats collected just before the first injection tested negative in the ELISA and WB. Inversely, all of them were found to be transitory positive with the C-ELISA after BTV vaccination. Two peaks of reactivity occurred at day 8 following the first (BTV-8) and second (BTV-8/1) injections, with 19 out of 21 animals (90%) testing positive. Subsequently, the reactivity levels of all sera slowly decreased and reached the negative range within a period of 6 months following the first injection. The reactivity levels of sera in the L-ELISA also revealed two peaks at day 8 following the first two injections, including 6 (29%) and 9 (43%) positive serum samples, respectively. Fifteen animals (71%) were positive at least once during a period of 6 months following the first injection. However, these sera produced weak positive responses, unlike those produced by the sera collected from the two CAEV-infected goats. Seven uninfected goats were inconsistently found positive between days 8 and 131 following the third infection. The statuses of all animals for CAEV infection were assayed at the end of the investigation using two confirmatory tests, whole-virus WB and a nested PCR targeting either the gag or env genes (3). Only the two CAEV-infected goats tested positive (data not shown).

| No. of days | Injection | No. of positive samples/total no. of samples (%) | C-ELISA | L-ELISA | P-ELISA | E-ELISA |
|------------|-----------|-----------------------------------------------|---------|---------|---------|---------|
| 21         | BTV-8     | 0/21 (0)                                      | NA      | NA      | NA      | 0/21 (0) |
| 31         | BTV-8     | 13/21 (62)                                    | 0/21 (0) | 0/21 (0) | 0/21 (0) | 0/21 (0) |
| 47         | BTV-8     | 0/21 (0)                                      | 0/21 (0) | 0/21 (0) | 0/21 (0) | 0/21 (0) |
| 101        | BTV-8     | 0/21 (0)                                      | 0/21 (0) | 0/21 (0) | 0/21 (0) | 0/21 (0) |
| 159        | BTV-8     | 0/21 (0)                                      | 0/21 (0) | 0/21 (0) | 0/21 (0) | 0/21 (0) |
| 226        | BTV-8     | 0/21 (0)                                      | 0/21 (0) | 0/21 (0) | 0/21 (0) | 0/21 (0) |
| 298        | BTV-8     | 0/21 (0)                                      | 0/21 (0) | 0/21 (0) | 0/21 (0) | 0/21 (0) |
| 326        | BTV-8     | 0/21 (0)                                      | 0/21 (0) | 0/21 (0) | 0/21 (0) | 0/21 (0) |
| 334        | BTV-8     | 19/21 (90)                                    | 6/21 (29) | 0/21 (0) | 0/21 (0) | 0/21 (0) |
| 345        | BTV-8     | 19/21 (90)                                    | 1/21 (5)  | 0/21 (0) | 0/21 (0) | 0/21 (0) |
| 354        | BTV-8     | 18/21 (86)                                    | 2/21 (10) | 0/21 (0) | 0/21 (0) | 0/21 (0) |
| 362        | BTV-8     | 19/21 (90)                                    | 9/21 (43) | 0/21 (0) | 0/21 (0) | 0/21 (0) |
| 373        | BTV-8     | 18/21 (86)                                    | 8/21 (38) | 0/21 (0) | 0/21 (0) | 0/21 (0) |
| 382        | BTV-8     | 18/21 (86)                                    | 3/21 (14) | 0/21 (0) | 0/21 (0) | 0/21 (0) |
| 390        | BTV-8     | 17/21 (81)                                    | 2/21 (10) | 0/21 (0) | 0/21 (0) | 0/21 (0) |
| 401        | BTV-8     | 17/21 (81)                                    | 2/21 (10) | 0/21 (0) | 0/21 (0) | 0/21 (0) |
| 411        | BTV-8     | 15/21 (71)                                    | 2/21 (10) | 0/21 (0) | 0/21 (0) | 0/21 (0) |
| 422        | BTV-8     | 13/21 (62)                                    | 2/21 (10) | 0/21 (0) | 0/21 (0) | 0/21 (0) |
| 432        | BTV-8     | 9/21 (43)                                     | 2/21 (10) | 0/21 (0) | 0/21 (0) | 0/21 (0) |
| 443        | BTV-8     | 9/21 (43)                                     | 3/21 (14) | 0/21 (0) | 0/21 (0) | 0/21 (0) |
| 453        | BTV-8     | 7/21 (33)                                     | 3/21 (14) | 0/21 (0) | 0/21 (0) | 0/21 (0) |
| 464        | BTV-8     | 8/21 (38)                                     | 0/21 (0) | 0/21 (0) | 0/21 (0) | 0/21 (0) |
| 474        | BTV-8     | 3/21 (14)                                     | 1/21 (5)  | 0/21 (0) | 0/21 (0) | 0/21 (0) |
| 485        | BTV-8     | 2/21 (10)                                     | 1/21 (5)  | 0/21 (0) | 0/21 (0) | 0/21 (0) |
| 499        | BTV-8     | 2/21 (10)                                     | 2/21 (10) | 0/21 (0) | 0/21 (0) | 0/21 (0) |
| 513        | BTV-8     | 0/21 (0)                                      | 4/21 (19) | 0/21 (0) | 0/21 (0) | 0/21 (0) |

* Plasma and serum samples from SRLV-uninfected goats were collected over the first (BTV-8) and second (BTV-1/8) vaccinations, respectively, and tested in different SRLV ELISAs based on antigens produced in either eukaryotic (C-ELISA and L-ELISA) or prokaryotic (P-ELISA and E-ELISA) systems. NA, not available.
In this study, we demonstrated that vaccination with inactivated BTV preparations in goats may temporarily affect the specificity of SRLV ELISAs based on antigens produced in eukaryotic cell cultures, including a whole-virus indirect ELISA (C-ELISA) and a competitive ELISA (L-ELISA) measuring the displacement of an anti-gp135 monoclonal antibody. We showed that increased reactivity in these assays following vaccination was correlated with higher reactivity of sera against the protein content of cells (BHK-21 cell line) used for the production of BTV vaccine. Moreover, the reactivity of sera against the BHK-21 cell lysate was enhanced after a second vaccination and was associated with an extended time scale of false-positive responses. Indeed, primary vaccination induced a subsequent increase of reactivity that did not exceed 3 months, whereas false-positive responses were detected over a period of 6 months following a second vaccination. All together, these results clearly indicated that false-positive responses in the SRLV ELISA following BTV vaccination are elicited from nonspecific proteins rather than cross-reactivity toward the viral envelope glycoprotein. In this regard, the C-ELISA and L-ELISA are established upon antigens expressed in a prokaryotic system (16). The clear discrepancy of results between the two sets of ELISAs (eukaryotic versus prokaryotic systems) supported the hypothesis that false-positive responses in the SRLV ELISA could be induced by immune responses directed against nonspecific proteins derived from the BHK-21 cell cultures used for the production of the BTV vaccine. To determine whether antibody reactivity toward proteins derived from BHK-21 cells was enhanced following BTV vaccination, selected samples obtained from two uninfected goats tested as seropositive after both the first and second vaccinations were analyzed in immunoblots against a lysate of BHK-21 cells. For this purpose, the cell lysate was fractionated by electrophoresis on a 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel, and proteins were transferred to a polivinyldiene difluoride membrane. Sera collected at different times (days –21, +47, +326, +362, +422, and +513) over the period of BTV vaccination were tested at a dilution of 1:100. The membrane was then incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody and developed by chemiluminescence. As shown in Fig. 3, the reactivity of sera in immunoblots was directly correlated to the optical density in the ELISA and temporarily increased following each BTV vaccination.

In attempt to characterize the false-positive ELISA responses following the BTV vaccination in goats, all sera were analyzed using two other commercially available SRLV ELISAs, the Pourquier CAEV/MVV ELISA (P-ELISA) from Pourquier/Idexx Laboratories and the ELITEST MVV ELISA (E-ELISA) from Hyphen BioMed Laboratories. These ELISAs are indirect assays based on the combination of an immunogenic peptide of the gp46 transmembrane glycoprotein with a recombinant p25 Gag protein. As shown in Table 1, the 21 uninfected goats were found to be negative over the period of investigation. Inversely, the two CAEV-infected goats were consistently found to be positive in both ELISAs (data not shown). The clear discrepancy of results between the different tests could result from the potential ability of the BTV vaccine to induce antibodies cross-reacting with the surface unit glycoprotein (gp135) of SRLV, since this viral protein was included as antigen in the C-ELISA and L-ELISA but was missing in the P-ELISA and E-ELISA. To address this possibility, all samples corresponding to the peaks of ELISA reactivity (days 8 and 36 after the second vaccination) were tested by a commercially available AGID test (Institut Pourquier, France) based on the detection of antibodies raised against the gp135 of SRLV. While sera obtained from the two CAEV-infected goats were found to be positive for anti-gp135 antibodies, sera obtained from all uninfected goats tested negative by AGID (data not shown). All together, these results indicated that false positivity in the SRLV ELISA following BTV vaccination was more likely related to immune responses directed against nonspecific proteins rather than cross-reactivity toward the viral envelope glycoprotein. In this regard, the C-ELISA and L-ELISA are established upon antigens which are produced in eukaryotic cell cultures (6, 19), whereas the P-ELISA and E-ELISA are based on antigens expressed in a prokaryotic system (16). The clear discrepancy of results between the two sets of ELISAs (eukaryotic versus prokaryotic systems) supported the hypothesis that false-positive responses in the SRLV ELISA could be induced by immune responses directed against nonspecific proteins derived from the BHK-21 cell cultures used for the production of the BTV vaccine. To determine whether antibody reactivity toward proteins derived from BHK-21 cells was enhanced following BTV vaccination, selected samples obtained from two uninfected goats tested as seropositive after both the first and second vaccinations were analyzed in immunoblots against a lysate of BHK-21 cells. For this purpose, the cell lysate was fractionated by electrophoresis on a 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel, and proteins were transferred to a polivinyldiene difluoride membrane. Sera collected at different times (days –21, +47, +326, +362, +422, and +513) over the period of BTV vaccination were tested at a dilution of 1:100. The membrane was then incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody and developed by chemiluminescence. As shown in Fig. 3, the reactivity of sera in immunoblots was directly correlated to the optical density in the ELISA and temporarily increased following each BTV vaccination.

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react with SRLV proteins, since the drawback in SRLV serology is the interference with serological testing due to the induction of immune responses against contaminating proteins derived from mammalian cell substrates, low purity levels of antigens in vaccine production may potentially result in the accidental introduction of undesirable agents. There are several examples of vaccine contamination by extraneous virus, for example, contamination of human and veterinary vaccines by simian virus 40 (SV40) from monkey primary renal and pestiviruses from fetal calf serum, respectively. Additionally, several vaccines are manufactured by using animal cell lines that are known to harbor replication-competent endogenous retroviruses which may be transmitted to the recipient. Finally, contamination of vaccines by residual host DNA containing activated oncogenes and/or latent infectious viral genomes might potentially induce oncogenic or infective events. Considering all these potential sources of vaccine contamination, the production of highly purified antigens is required to provide a better safety profile. However, the methods suitable for the industrial production of highly purified vaccine are costly and time-consuming. These requirements are somewhat difficult to achieve in the context of a severe epizootic such as that of bluetongue disease, leading to the implementation of a large-scale compulsory vaccination campaign.

In conclusion, CAEV and MVV are antigenically related viruses which are detected using the same diagnostic tools. Therefore, it is likely that the BTV vaccination campaign is responsible for the false-positive ELISA responses in MVV-free flocks. Studies assessing the impact of BTV vaccination on the reliability of SRLV ELISAs in sheep are under way. It is advisable to carry out appropriate ELISAs for the monitoring of SRLV infection in sheep and goat flocks in which BTV vaccination took place. ELISAs based on either synthetic peptides or recombinant antigens produced in prokaryotic systems are suitable to counteract the interference of BTV vaccination on serological testing of SRLV infections.

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prokaryotic system (P-ELISA and E-ELISA). While the loss of specificity in the indirect ELISA may be explained by the low purity of whole-virus preparations, the mechanisms responsible for the weak decrease of specificity in the competitive ELISA remain unclear. In this assay, soluble gp135 from the medium of CAEV-infected cell cultures was captured on ELISA plates coated with an anti-gp135 monoclonal antibody, and the reactivity of sera with gp135 was determined by inhibition of binding by a second monoclonal antibody conjugated with horseradish peroxidase. Therefore, it is not excluded that binding of antibodies to nonspecific proteins absorbed onto the ELISA plate impairs the recognition of gp135 by the second monoclonal antibody.

While the first and second vaccinations against BTV-8 were followed by transitory false-positive responses in SRLV ELISA, there was no evidence for increased reactivity after the injection of the BTV-1 dose alone, arguing in favor of the minor effect of the BTV-1 vaccine on the specificity of the SRLV ELISA. However, the content of foreign materials in vaccine preparations probably depends on the process used for vaccine production, and further experiments are required to determine whether the different BTV vaccine preparations have the same propensity to affect the serological testing of SRLV infection. In contrast, it is unlikely that natural infection with BTV induced the production of antibodies which cross-react with SRLV proteins, since the drawback in SRLV serological testing was reported only after the vaccination campaign but not during the major epidemic of bluetongue in northwest Europe in 2007.

In addition to the interference with serological testing due to the induction of immune responses against contaminating proteins derived from mammalian cell substrates, low purity levels of antigens in vaccine production may potentially result in the accidental introduction of undesirable agents. There are several examples of vaccine contamination by extraneous virus, for example, contamination of human and veterinary vaccines by simian virus 40 (SV40) from monkey primary renal and pestiviruses from fetal calf serum, respectively. Additionally, several vaccines are manufactured by using animal cell lines that are known to harbor replication-competent endogenous retroviruses which may be transmitted to the recipient. Finally, contamination of vaccines by residual host DNA containing activated oncogenes and/or latent infectious viral genomes might potentially induce oncogenic or infective events. Considering all these potential sources of vaccine contamination, the production of highly purified antigens is required to provide a better safety profile. However, the methods suitable for the industrial production of highly purified vaccine are costly and time-consuming. These requirements are somewhat difficult to achieve in the context of a severe epizootic such as that of bluetongue disease, leading to the implementation of a large-scale compulsory vaccination campaign.

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