Evaluation of Newly Developed Immunoperoxidase Monolayer Assays for Detection of Antibodies against Bovine Herpesvirus 4

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Received 7 January 1999/Accepted 25 February 1999

Bovine herpesvirus 4 (BHV4), a group of virus strains belonging to the Gammaherpesvirinae (17), is distributed worldwide, and BHV4 strains have been isolated from cattle with a variety of clinical symptoms (8, 12, 14, 20, 23, 24). For antibody detection, a sensitive test is required because the humoral immune response of cattle to BHV4 infections is characterized by a low neutralizing antibody response, or even by the absence of neutralizing antibodies (21). Other methods have been developed to detect BHV4 antibodies in serum, such as complement fixation (9), the indirect fluorescent-antibody test (IFAT) (18), and the indirect enzyme-linked immunosorbent assay (iELISA) (7). According to Guo et al. (9), the complement fixation test was less sensitive than the iELISA for the detection of immunoglobulin G (IgG) to BHV4. The IFAT and the iELISA are the most common tests to be used for detection of BHV4 antibodies, but the IFAT is laborious, particularly if quantitative results are required (7). Also, cross-reactions between BHV4 and other herpesviruses have been recorded by IFAT (11, 16). Edwards and Newman (7) reported that the iELISA appeared to be as sensitive as the IFAT, but these results were based only on sera collected from experimentally infected cattle. Until now, no thorough validation of any BHV4 ELISA has been published. Because there is a need for an easy test to detect BHV4 antibodies, we developed an immunoperoxidase monolayer assay (IPMA). IPMAs have proven to be an easy method and a valuable tool for the diagnosis of several infectious diseases (19); for instance, it is a common method for the detection of antibodies against porcine reproductive and respiratory syndrome virus in pigs (10).

The purpose of this study was to evaluate newly developed BHV4 IPMAs which are based on the American BHV4 reference strain, DN-599 (13), and on LVR 140, the Belgian BHV4 reference strain (26), which belongs to the European Mover 33/63-like group (22). The results of the IPMAs were compared with the results of a BHV4 iELISA. Data on the prevalence of BHV4 antibodies in Dutch cattle are also presented.

MATERIALS AND METHODS

BHV4 IPMA. (i) Preparation of BHV4 IPMA plates. Trypsinized embryonic bovine trachea (EBTr) cells were resuspended in Earle’s minimal essential medium cell culture medium containing 10% horse serum and 0.5% antibiotic mix (antibiotic stock solution containing 107 IU of penicillin G, 8.6 × 106 IU of streptomycin, 1% kanamycin, and 5 × 106 IU of nystatin per liter of aquades) and seeded into wells of a 96-well cell culture plate (Greiner). Each well contained 100 μl of an EBTr cell suspension of approximately 7.5 × 103 cells/ml. The plates were placed in a humidified incubator at 37°C with 5% CO2. After 3 to 4 days, when the monolayer was 100% confluent, the cell culture medium was discarded and the cells were infected with 25 μl (containing 105 50% tissue culture infective doses/ml) of the BHV4 DN-599 strain (ATCC VR631) or the BHV4 LVR 140 strain (kindly provided by E. Thiry). After 1 h at 37°C (5% CO2), a volume of 75 μl of EMEM (containing 2% horse serum and 0.5% antibiotic mix) was added to each well. When a cytopathogenic effect started to appear, the incubation was stopped. The virus-cell culture medium was discarded, and a volume of 150 μl of 4% formaldehyde in phosphate-buffered saline (PBS) was added to the wells for 10 min at 18 to 25°C. The fluid was poured off, and again a volume of 150 μl of 4% formaldehyde in PBS (fixative) was added to the wells. The plates were sealed and stored at 4°C.

(ii) Performance of BHV4 IPMA. Prior to use, the fixative was removed and the plates were washed once with 100 μl/well of a 2% Triton X-100 solution (Sigma) in PBS. To reduce nonspecific reactions, the plates were first incubated with a volume of 100 μl of 2% Triton X-100 in PBS for 60 min at 37°C. After the Triton X-100 solution was discarded, the plates were incubated with 75 μl of PBS (containing 10% horse serum/well) for 60 min at 37°C. Serum test samples were diluted 1:20 in IPMA buffer (38.5 g of NaCl, 2% Tween 80, 0.1% NaN3, and 10% horse serum per 1 liter of PBS). After the preincubation, the PBS-horse serum solution was discarded and a volume of 75 μl of the 1:20 prediluted serum sample was added to the wells and incubated at 37°C for 1 h. The plates were washed six times in wash solution (PBS containing 1% Tween 80 [Merck]). A volume of 75 μl of the conjugate solution was added to the wells (1:200 dilution of horseradish peroxidase labeled rabbit anti-bovine immunoglobulin [Dakopatts] in IPMA buffer).
the binding of serum antibodies to BHV4 antigens. (B) Negative reactions are characterized by an intense red-brown staining mainly of the nuclei of EBTr cells, indicating the absence of color.

The results were read under a light microscope by at least two persons, independently of each other. The serum samples were tested if the interpretations of the two persons were not the same. Positive reactions were characterized by an intense red-brown staining mainly of the nuclei of the EBTr cells, indicating the binding of antibodies to BHV4 antigens. Negative reactions are characterized by red-brown staining mainly of the nuclei of BHV4-infected EBTr cells, indicating the absence of color.

Evaluation of the BHV4 IPMAs. (i) Specificity. No positive reactions were observed in the BHV4 IPMAs and the BHV4 iELISA with the monospecific serum samples containing high antibody levels directed against BHV1 (number of serum samples [n] = 5), BHV2 (n = 1), bovine respiratory syncytial virus (n = 3), parainfluenza virus type 3 (n = 3), bovine leukemia virus (n = 2), bovine viral diarrhea virus (n = 3), and coronavirus (n = 4). The specificities of the BHV4 IPMAs and the BHV4 iELISA were also determined by testing 69 individual serum samples collected from 69 specified-pathogen-free (SPF) cattle (cattle born by caesarean section, held in isolation stables, and raised on a controlled diet). The detection limit was defined as the reciprocal of the highest dilution giving a positive reaction.

Experimental infection. Two SPF calves (4797 and 4798), 3 weeks of age, were intranasally inoculated with 10 ml of 10^6.5 50% tissue culture infective doses of BHV4 DN-599 (ATCC VR631; bovine viral diarrhoea virus and Mycoplasma bovis) free)/ml. Pre- and postinoculation serum samples were collected and tested for BHV4 antibodies. The serum samples were tested in the BHV4 IPMAs and the BHV4 iELISA in a 1:20 dilution. Positive serum samples were titrated in serial twofold dilutions, starting at a dilution of 1:20.

Reproducibility. The reproducibility of the BHV4 tests was calculated from the results of duplicate tests by use of the Kappa test (2). Kappa (κ) was defined as the quotient of the observed proportion of agreement beyond chance and the maximal proportion of agreement. Therefore, serum samples (n = 150) of six randomly chosen BHV4-positive herds of the field study were tested twice on two different occasions, at least 2 months apart, using new IPMA reagents and ELISA kits with different lot numbers.

Field sera. Field sera were used to compare the newly developed BHV4 IPMAs with the iELISA and to estimate the BHV4 seroprevalence in Dutch cattle. For that purpose, a total of 750 serum samples were collected at random from 30 randomly chosen Dutch herds (25 serum samples per herd). These herds had participated in a field study for BHV1 marker vaccine efficacy (3). The date of birth of each animal was recorded to estimate the BHV4 seroprevalence in Dutch cattle at different ages.

Statistical analyses and data processing. Statistical analysis was performed on the data in Table 1 by the Friedman nonparametric two-way analysis of variance test, followed by the Wilcoxon signed-rank test (one-sided test) for pairwise comparison of both IPMAs with the iELISA. The data were processed with Statistica for Windows, version 2.0.

RESULTS
and older showed the highest BHV4 seroprevalence: 43% of the sera contained antibodies to BHV4. The lowest level of BHV4 antibodies (6 to 7%) was found in the group of 0.5- to 2-year-old cattle. In young calves (between 0 and 6 months old) the BHV4 seroprevalence was 31%. Figure 2 shows only the results of the BHV4 LVR 140 IPMA for BHV4 seroprevalence in Dutch cattle at different ages; the same patterns were observed with the BHV4 DN-599 IPMA and the BHV4 iELISA.

**DISCUSSION**

Our study shows that the BHV4 DN-599 and BHV4 LVR 140 IPMAs can be used for the screening of herds for BHV4 antibodies as an alternative to the BHV4 iELISA. Based on the specificity, detection limit, results from early-infection sera collected from calves, and reproducibility, the newly developed BHV4 IPMAs are reliable practical tests for the detection of BHV4 antibodies. In this evaluation study, the BHV4 IPMA specificities (100%) are comparable with those of the BHV4 iELISA (100%). The data on the detection limits (analytical sensitivity) and the experimental infection sera (diagnostic sensitivity) for both BHV4 IPMAs are even better than those of the BHV4 iELISA. The geometrical mean antibody titers (detection limits) of the BHV4 IPMAs were twice as high as the geometrical mean antibody titer of the BHV4 iELISA, and the differences between the BHV4 antibody titers of both BHV4 IPMAs and those of the BHV4 iELISA were statistically significant.

TABLE 2. Antibody responses against BHV4 in experimentally infected calves as determined in the BHV4 IPMAs and the BHV4 iELISA

| Day postinfection | BHV4 antibody titer | IPMA | iELISA |
|-------------------|---------------------|------|-------|
| 0                 | &lt;20               | 4797 | 4797  |
| 7                 | &lt;20               | 4797 | 4797  |
| 11                | &lt;20               | 4797 | 4797  |
| 14                | &lt;20               | 4797 | 4797  |
| 16                | &lt;20               | 4797 | 4797  |
| 18                | 20                  | 40   | 40    |
| 21                | 80                  | 80   | 80    |
| 23                | 160                 | 160  | 160   |
| 30                | 320                 | 320  | 320   |
| 37                | 640                 | 320  | 320   |
| 44                | 640                 | 320  | 320   |

TABLE 3. Concurrence of BHV4 IPMA and BHV4 iELISA results for field sera (n = 750)

| IPMA               | Result | No. of samples |
|--------------------|--------|----------------|
| BHV4 DN-599        | +      | 107 13         |
| iELISA             | –      | 27 603         |
| BHV4 LVR 140       | +      | 119 16         |
| iELISA             | –      | 15 600         |

* +, positive; –, negative.

* Titer of &lt;100 was considered negative; titer of &ge;100 was considered positive.

* Titer of &lt;20 was considered negative; titer of &ge;20 was considered positive.
positive animals was recorded within the group of 2- to 3-year-old cattle. Hence, it is likely that most cattle become infected after introduction into the dairy herd. Whether there is a relation between this finding and that of Czaplicki and Thiry (4), who reported an association between BHV4 seropositivity and abortion in cows, is unknown.

Although the BHV4 group comprises a collection of antigenically closely related isolates (1), and no major antigenic differences between BHV4 isolates have been demonstrated by cross-serological analysis with polyvalent antisera (5), the evaluation of the newly developed BHV4 IPMA was based on two different BHV4 strains. The IPMAs were performed with the American BHV4 DN-599 reference strain and the Belgian LVR 140 reference strain, which belongs to the European Movar 33/63-like group (22), because the use of monoclonal antibodies showed some differences in the antigenic patterns of BHV4 isolates (6) and BHV4 strains can be isolated in many countries all over the world. Dubuisson et al. (6) confirmed the close antigenic relationships between BHV4 isolates by comparison of field isolates with BHV4 monoclonal antibodies, but some monoclonal antibodies recognized all BHV4 isolates while others allowed differentiation among them. Our data support the finding that there could be some minor differences in the antigenic patterns of BHV4 isolates, because the results, obtained with field sera, showed some slight differences between the BHV4 DN-599 IPMA and the BHV4 LVR 140 IPMA. Fifteen field serum samples reacted positive in the BHV4 LVR 140 IPMA, while these sera did not react in the BHV4 DN-599 IPMA. Thirteen of the 15 sera also reacted positive in the BHV4 iELISA, in which the plates were coated with a BHV4 LVR 140-cell suspension lysate. Differences in affinities of certain immunoglobulins of cattle for the different BHV4 strains could be an explanation, but another explanation could be the differences in antigenic expression or differences in the exposure of certain BHV4 antigens. Probably the antigenic expression and antigen exposure of Dutch BHV4 strains are more related to those of BHV4 LVR 140 than to those of BHV4 DN-599.

The BHV4 IPMA has several advantages compared to the BHV4 iELISA, e.g., this study shows better analytical sensitivity results and the IPMA can be more easily adapted to the BHV4 strain of choice (for example, the BHV4 strain which has been isolated in the area of interest) while the commercial BHV4 iELISA is based on the use of BHV4 DN-599. This study also shows that, in comparison to the BHV4 iELISA, the BHV4 IPMA, which is based on the detection of all bovine immunoglobulin subclasses, is the test of choice for the early detection of BHV4 antibodies in bovine serum samples. A disadvantage of the IPMA is that it depends on subjective readings of test results whereas the iELISA is more objective because of the use of automatic readers. IPMA test results need to be interpreted by at least two trained persons to reduce the subjectivity of readings of test results (as indicated in Materials and Methods).

In conclusion, the BHV4 IPMA is a reliable practical test for the screening of cattle for BHV4 antibodies, and it is a useful alternative to iELISA for detecting BHV4 antibodies in cattle.

ACKNOWLEDGMENTS

We gratefully acknowledge J. de Bree for statistical advice, E. Thiry for providing the BHV4 LVR 140 strain, and G. Czaplicki and E. Van Opdenbosch for providing BHV4 sera.

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