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Flow Cytometric Detection of *Mycobacterium avium* subsp. *paratuberculosis*-Specific Antibodies in Experimentally Infected and Naturally Exposed Calves

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A desirable test to diagnose infections with *Mycobacterium avium* subsp. *paratuberculosis* facilitates identification of infected cattle prior to the state of *M. avium* subsp. *paratuberculosis* shedding. This study aimed at adjusting a flow cytometry (FC)-based assay, using intact *M. avium* subsp. *paratuberculosis* bacteria as the antigen, for diagnosis of *M. avium* subsp. *paratuberculosis* infections in calves. Serum samples were collected from experimentally infected ($n = 12$) and naturally exposed ($n = 32$) calves. Samples from five calves from positive dams were analyzed to determine the dynamics of maternal antibodies. Samples from adult cattle with defined infection status served as the standard (18 *M. avium* subsp. *paratuberculosis* shedders, 22 *M. avium* subsp. *paratuberculosis* free). After preadsorption with *Mycobacterium phlei*, sera were incubated with *M. avium* subsp. *paratuberculosis* and *M. avium* subsp. *avium* bacterial suspensions, respectively, followed by the separate detection of bovine IgG, IgG1, IgG2, and IgM attached to the bacterial surface. *M. avium* subsp. *paratuberculosis*-specific sample/positive (S/P) ratios were compared to enzyme-linked immunosorbent assay (ELISA) S/P ratios. In adult cattle, the FC assay for IgG1 had a sensitivity of 78% at a specificity of 100%. Maternally acquired antibodies could be detected in calves up to 121 days of life. While all but two sera taken at day 100 ± 10 postnatum from naturally exposed calves tested negative, elevated S/P ratios (IgG and IgG1) became detectable from 44 and 46 weeks postinoculation onwards in two calves infected experimentally. Even with the optimized FC assay, *M. avium* subsp. *paratuberculosis*-specific antibodies can only occasionally be detected in infected calves less than 12 months of age. The failure to detect such antibodies apparently reflects the distinct immunobiology of *M. avium* subsp. *paratuberculosis* infections rather than methodological constraints.

Paratuberculosis, also referred to as Johne's disease, is a chronic intestinal infectious disease of ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis*. Paratuberculosis has been recognized as a major herd problem in dairy cattle husbandry (1). Calves, in their first weeks of life in particular, are at the highest risk of becoming infected with *M. avium* subsp. *paratuberculosis*. The fecal-oral route is considered the primary route of transmission from an infected mother to its offspring. Paratuberculosis proceeds very slowly after a long incubation period. Although infected early in life, the first signs of illness are usually not seen until animals are 2 to 6 years old (2). A major obstacle for counteracting the spread of infection is the fact that infected animals begin to shed the pathogen in large numbers with their feces long before symptoms occur. Being capable of reliably identifying *M. avium* subsp. *paratuberculosis*-infected animals even before reaching the state of shedding would be a significant step forward to the establishment of successful sanitizing programs.

In general, identification of *M. avium* subsp. *paratuberculosis*-infected cattle is a difficult task. Culture detection of *M. avium* subsp. *paratuberculosis* in feces remains the "gold standard" of *M. avium* subsp. *paratuberculosis* diagnosis *intra vitam* but is hampered by long incubation periods, high contamination rates with other fecal bacteria and fungi, and high costs (3). Intermittent shedding patterns and excretion of *M. avium* subsp. *paratuberculosis* in numbers at the limits of detection significantly impair the sensitivity of culture methods. Indirect assays would represent reasonable alternatives. Particularly enzyme-linked immunosor-

bent assays (ELISAs) to detect *M. avium* subsp. *paratuberculosis*-specific antibodies in blood or milk samples are considered methods of choice for screening dairy herds. The main advantages are low costs, high-throughput options, and availability of results within days. However, currently available ELISAs exhibit low sensitivities between 17 and 56% at the individual animal level and are applicable in adult cattle only (4).

Eda et al. (5) introduced a sensitive serological assay following a novel approach deploying flow cytometry (FC). In this FC assay, intact *M. avium* subsp. *paratuberculosis* cells serve as the test antigen and measuring particle at the same time. The complete repertoire of unmodified *M. avium* subsp. *paratuberculosis* surface antigens is available for recognition by antibodies in bovine serum samples in this setting. In naturally infected adult cattle, the FC assay detected *M. avium* subsp. *paratuberculosis* infections 6 to 44 months earlier than fecal culture. Retaining a high specificity, this method allowed detection of *M. avium* subsp. *paratuberculosis*-

specific antibodies in calves as early as 170 days after experimental infection (5).

Due to the intracellular persistence of *M. avium* subsp. *paratuberculosis* in macrophages, a cell-mediated immune response is the key mechanism to limit the infection at an early stage. Once the *M. avium* subsp. *paratuberculosis*-infected macrophage becomes activated, it is believed to predominately stimulate specific T helper cells of the T_h1 type (6). These T cells then release cytokines, such as interleukin-2 (IL-2) and gamma interferon (IFN-γ) (7, 8). IFN-γ inhibits proliferation of T_h2 cells, which are the source of IL-4, IL-5, and IL-10 and primarily promote the onset of humoral immune responses. During progression of *M. avium* subsp. *paratuberculosis*-infected cattle from the asymptomatic carrier (and shedding) state to the state of clinical paratuberculosis, the decrease in cell-mediated immune responses and the concomitant enhancement of humoral responses reflect a switch from a T_h1- to a T_h2-type cell response (6, 9). Although the T_h2 response appears to be associated with later stages of *M. avium* subsp. *paratuberculosis* infection, some investigators found *M. avium* subsp. *paratuberculosis*-specific antibodies in cattle less than 1 year of age—i.e., as early as 134 days (10) and 170 days (5) postinoculation.

T_h1- and T_h2-related cytokines also determine the Ig class or subclass synthesized by plasma cells. In cattle, T_h1-related IFN-γ and IL-2 enforce production of IgG2 and IgM, whereas T_h2-related IL-4 induces a switch from IgM to IgG1 and IgE (11–13). Given the predominance of T_h1 immune responses in the early phase of an *M. avium* subsp. *paratuberculosis* infection, we hypothesized that *M. avium* subsp. *paratuberculosis*-infected calves produce IgG2 rather than IgG1. Accordingly, Koets et al. (14) showed that IgG2 titers, but not IgG1 titers, were significantly higher in asymptomatic (i.e., early stage) shedders than in uninfected control cows. Notably, this effect became apparent when complex bacterial cell antigens, like purified protein derivative (PPD) of *M. avium* subsp. *paratuberculosis*, were used as the test antigen but not when purified bacterial components, such as heat shock proteins or lipoarabinomannan (LAM), were used.

In order to address the urgent needs for a reliable diagnostic procedure to detect *M. avium* subsp. *paratuberculosis*-specific humoral immune responses in calves, we combined the FC assay introduced by Eda et al. (5) with the differential detection of anti-*M. avium* subsp. *paratuberculosis* Ig subclasses to further increase the sensitivity of this approach. We also added a preadsorption step with *Mycobacterium phlei* and used reactions against *M. avium* subsp. *avium*, a pathogen closely related to *M. avium* subsp. *paratuberculosis*, when calculating *M. avium* subsp. *paratuberculosis*-specific sample/positive (S/P) ratios to ensure sufficient specificity. We evaluated this modified FC assay with serum samples taken from naturally infected cows and from calves experimentally infected with or naturally exposed to *M. avium* subsp. *paratuberculosis*.

MATERIALS AND METHODS

Animals and study design. (i) Adult cattle. *M. avium* subsp. *paratuberculosis*-negative cows (*n* = 22) were selected from herds in which all female cattle aged over 24 months had tested *M. avium* subsp. *paratuberculosis* negative using serological assays (Svanovir-ELISA [ELISA C; Svanova, Uppsala, Sweden] and Pourquier-ELISA [ELISA B; IDEXX GmbH, Ludwigsburg, Germany]) as well as fecal culture and a real-time PCR assay (15). Animals from other herds, proven to shed *M. avium* subsp. *paratuberculosis* in their feces either by PCR or culture, were selected as *M. avium* subsp. *paratuberculosis*-positive cows (*n* = 18).

(ii) *M. avium* subsp. *paratuberculosis*- and mock-infected calves. Eighteen German-Holstein calves were purchased from four different *M. avium* subsp. *paratuberculosis*-free herds (defined as described in the previous paragraph). Six calves served as mock-infected animals (control group, calves E1 to E6). Twelve calves were separated into two groups and infected with *M. avium* subsp. *paratuberculosis* subsequently (group I, calves E7 to E12; group II, calves E13 to E18). Experiments with the three groups started at different time points with ~8 months between the single groups, starting with the control group. Each calf was supposed to be sampled up to 55 weeks postinoculation (wpi). On days 10, 12, and 14 postnatum (dpn), calves of groups I and II were challenged orally with a total amount of 1×10^9 CFU of *M. avium* subsp. *paratuberculosis* reference strain K10 (see below). Calves of the control group received mock inocula (the same volume of Middlebrook 7H9 [MB 7H9] medium in 2 liters of milk replacer but without *M. avium* subsp. *paratuberculosis*). To confirm a successful infection with *M. avium* subsp. *paratuberculosis*, biopsy specimens from intestinal lymph nodes were taken 14, 30, 90, and 170 days postinoculation (dpi). *M. avium* subsp. *paratuberculosis* could be detected in ileocecal and jejunal lymph nodes by culture or PCR after 90 dpi in all calves from *M. avium* subsp. *paratuberculosis*-infected groups I and II (data not shown). Serum samples were collected monthly during the observation period and tested for antibodies against *M. avium* subsp. *paratuberculosis* by FC assay and ELISA B. Calf E9 succumbed to a severe peritonitis, had to be euthanized at the age of 253 days, and was sampled up to 34 wpi only. Approximately 12 months after inoculation, all remaining calves were euthanized and subsequently submitted to a detailed necropsy procedure, including extensive sampling of various tissues from different locations (unpublished data).

The study was carried out in strict accordance with European and German laws for the care and use of animals. All animal experiments were approved by the local authorities: Regierungspraesidium, Giessen, Germany, permit no. V 54-19 c 20-25 (1) GI 18/15-Nr. 43/2007. The number of calves enrolled in this study was the minimum required for reliable results.

(iii) Naturally *M. avium* subsp. *paratuberculosis*-exposed calves. Serum samples were collected between 90 and 110 dpn (100 ± 10 dpn) and between 170 and 230 dpn (200 ± 30 dpn) from 15 calves in three *M. avium* subsp. *paratuberculosis*-free herds and from 32 calves in 8 herds where paratuberculosis had been diagnosed previously by both clinical examination and fecal culture. At 200 ± 30 dpn, only 30 calves in paratuberculosis herds could be sampled since two calves had died from coccidiosis. At 100 ± 10 dpn, biopsy specimens from intestinal lymph nodes were taken from all calves and tested for the presence of *M. avium* subsp. *paratuberculosis* by real-time PCR or culture at the Institute of Veterinary Food Science, Giessen, Germany. *M. avium* subsp. *paratuberculosis* was detected by real-time PCR and/or culture in biopsy specimens of ileocaecal and/or jejunal lymph nodes from three calves in herds with paratuberculosis.

(iv) Calves from *M. avium* subsp. *paratuberculosis*-seropositive dams. Dairy cows from one herd with confirmed cases of paratuberculosis were classified into *M. avium* subsp. *paratuberculosis*-seropositive and -seronegative dams by the results of the FC assay. Serum samples were taken every 6 weeks from five calves (M1 to M5) of FC assay-positive dams and from six calves (M6 to M11) of FC assay-negative dams, starting in the first 3 weeks of life up to an age of 200 days.

Mycobacterial antigens and inoculum preparation. Lyophilized cells of *M. avium* subsp. *paratuberculosis* strain K10 (ATCC BAA-968) were obtained from the American Type Culture Collection (Manassas, VA). Strains of *Mycobacterium avium* subsp. *avium* (DSMZ no. 44156) and *Mycobacterium phlei* (DSMZ no. 43239) were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ). All mycobacteria were grown in Middlebrook 7H9 (MB 7H9) medium, supplemented with 2 mg/ml of mycobactin J (Allied Monitor, Inc., Fayette, MO) and 10% oleic acid-albumin-dextrose-catalase complex (Becton, Dickin-

son, Heidelberg, Germany) plus 0.05% Tween 80 (Sigma-Aldrich Chemie GmbH, Munich, Germany).

M. avium subsp. *paratuberculosis* K10 was used as the challenge strain for the experimental infection study as well as the test antigen in the FC assay. For preparation of the inocula, *M. avium* subsp. *paratuberculosis* K10 cultures were harvested in the mid-log growth phase, supplemented with glycerin (15% final concentration), portioned into several aliquots, and frozen at −70°C to be used as seed material for each *M. avium* subsp. *paratuberculosis*-infected group. Approximately 10 weeks prior to the inoculation into calves of groups I and II, several aliquots of the seed material were subcultured in fresh MB 7H9 medium and incubated for 4 to 8 weeks at 37°C. Then, aliquots containing 1×10^9 living *M. avium* subsp. *paratuberculosis* bacteria in 5 ml MB 7H9 medium were prepared using the LIVE/DEAD BacLight bacterial viability and counting kit (Invitrogen GmbH, Karlsruhe, Germany). Aliquots were stored at −70°C. One day before inoculation, one aliquot was thawed for each calf and diluted with MB 7H9 medium to a final volume of 25 ml. This suspension was incubated overnight at 37°C, mixed with 2 liters of milk replacer the next day, and administered orally to the calf. F57 and IS*Mav*2 real-time PCR (15) and IS*900* nested PCR (16) were conducted to confirm that the seed material as well as the inoculum contained *M. avium* subsp. *paratuberculosis*.

Mycobacteria were used as test antigens (*M. avium* subsp. *avium* and *M. phlei*) and adsorptive agents (*M. phlei*) in the FC assay. To standardize the assay, one batch of each strain was produced. Mycobacteria were cultured in MB 7H9 medium and harvested in the mid-log growth phase with optical densities at 660 nm (OD_{660}) of 0.3 (*M. avium* subsp. *paratuberculosis* and *M. avium* subsp. *avium*) and of 0.1 (*M. phlei*) at 660 nm, respectively. Each mycobacterial suspension was supplemented with 16.6% glycerin and stored in several aliquots at −70°C.

FC analysis. Frozen aliquots of mycobacteria were thawed at 37°C and centrifuged with $4,000 \times g$ for 10 min at room temperature (RT). Pellets were resuspended in buffer A consisting of phosphate-buffered saline (PBS [pH 7.0]) containing 10% SuperBlock (Perbio Science Deutschland, Bonn, Germany) and 0.05% Tween 80 (Sigma-Aldrich Chemie GmbH, Munich, Germany). For use as an adsorbent, the *M. phlei* suspension was adjusted to an OD_{660} of 0.6, while *M. avium* subsp. *avium* and *M. phlei* test antigen suspensions were adjusted to an OD_{660} of 0.1. Preadsorption of serum samples was accomplished by incubating 2 µl of serum with 98 µl of *M. phlei* suspension for 1 h at RT. Then, serum samples were centrifuged with $16,000 \times g$ for 10 min at RT. The supernatants were collected and used for analysis. Wells of a V-shaped 96-well microtiter plate (Greiner Bio-One GmbH, Frickenhausen, Germany) were loaded with the *M. avium* subsp. *paratuberculosis* or *M. avium* subsp. *avium* test antigen suspension (100 µl/well). After centrifugation ($2,500 \times g$, 8 min, 4°C) each pellet was suspended in 100 µl of the *M. phlei*-preadsorbed supernatants of the test or control serum sample. After incubation for 1 h at RT, the plate was centrifuged at $2,500 \times g$ for 8 min at 4°C. Pellets were washed twice with buffer A and then resuspended in 50 µl buffer A containing fluorescein isothiocyanate (FITC)-labeled secondary antibody (sheep anti-bovine IgG [dilution 1:200], sheep anti-bovine IgG1 [dilution 1:200], sheep anti-bovine IgG2 [dilution 1:100], or sheep anti-bovine IgM [dilution 1:100] antibody, each from AbD Serotec, Düsseldorf, Germany) and incubated for another hour at RT in the dark. After centrifugation ($2,500 \times g$, 10 min, 4°C), bacteria were suspended in 100 µl PBS, transferred to tubes prefilled with 200 µl PBS, and subjected to flow cytometric analysis (FACSCalibur; Becton, Dickinson, Heidelberg, Germany). The CellQuest Pro software (Becton, Dickinson, Heidelberg, Germany) was used for data analysis. Data acquisition was terminated when 30,000 particles were acquired in a forward scatter (FSC) versus sideward scatter (SSC) plot defining the morphologically intact mycobacteria. Mean fluorescence intensities (MFI) of the FITC signal of the selected events were determined. For each sample, the difference of the MFI using *M. avium* subsp. *paratuberculosis* and *M. avium* subsp. *avium* as test antigens was calculated. Serum from an *M. avium* subsp. *paratuberculosis*-shedding cow in a herd with a history of paratuberculosis was used as the posi-

tive-control serum. The following formula was used to calculate the *M. avium* subsp. *paratuberculosis*-specific S/P ratio: S/P ratio (%) = [(*M. avium* subsp. *paratuberculosis* MFI_{serum} − *M. avium* subsp. *avium* MFI_{serum})/(*M. avium* subsp. *paratuberculosis* $MFI_{positive\ control}$ − *M. avium* subsp. *avium* $MFI_{positive\ control}$)] × 100.

ELISA. Results from the FC assay were compared to those obtained with the commercially available Pourquier ELISA Paratuberculosis (ELISA B; IDEXX GmbH, Ludwigsburg, Germany). Serum samples from adult cattle naturally infected with *M. avium* subsp. *paratuberculosis* were also tested with Svanovir Para-Tb ELISA (ELISA C; Svanova, Uppsala, Sweden) and Cattletype *M. avium* subsp. *paratuberculosis* Ab ELISA (ELISA A; Labor Diagnostik GmbH, Leipzig, Germany). All ELISAs were performed according to the instructions provided by the manufacturers.

Statistical analysis. Test results obtained in the FC assay and by ELISA testing of serum samples from six mock-infected calves (control group) were used to establish cutoffs between seronegative and inconclusive test results as well as inconclusive and seropositive results. These cutoff values were defined by calculating the 95th and 99th percentiles of all values obtained from the six control calves by using the respective FC assay format or ELISA. S/P ratios of ≥95th and <99th percentiles were interpreted as inconclusive; S/P ratios of ≥99th percentile were considered positive.

To compare the results of ELISAs and the FC assay, S/P ratios were subjected to receiver-operating characteristic (ROC) curve analysis (SPSS Statistic version 17; SPSS Inc., Chicago, IL). This method estimates the sensitivity and specificity of the assays at every possible cutoff and provides an overall measure of test accuracy as the area under the curve (AUC).

Prior to statistical analysis (SPSS Statistic 17.0, SPSS, Inc.) a logarithmic transformation of those data that were not normally distributed was performed. Student′s *t* test was used to determine whether *M. avium* subsp. *paratuberculosis*-specific S/P ratios of calves from paratuberculosis herds were significantly higher than those of calves in *M. avium* subsp. *paratuberculosis*-free herds.

RESULTS

Detection of *M. avium* subsp. *paratuberculosis*-specific antibodies in naturally infected adult cattle. Serum samples from 18 *M. avium* subsp. *paratuberculosis*-shedding and 22 *M. avium* subsp. *paratuberculosis*-free cows were tested by the FC assay for their content of *M. avium* subsp. *paratuberculosis*-specific IgG, IgG1, and IgG2, separately. Comparing the different immunoglobulin classes or subclasses, FC-based detection of IgG1 resulted in the highest sensitivity of 78% at a specificity of 100% (Fig. 1A). In a specificity range of ≤90.9%, the ROC curve of IgG1 detection was nearly identical to that of IgG detection. When detecting IgG2, the FC assay exhibited a sensitivity of only 44% at 100% specificity. With the same panel of defined serum samples, the sensitivities at 100% specificity of ELISA kits A, B, and C were 61%, 72%, and 39%, respectively (Fig. 1B). When applying the cutoffs recommended by the manufacturers, sensitivities at 100% specificity of the ELISA kits were even lower (61% for ELISA B and 28% for ELISAs A and C). The IgG1 detection by the FC assay had the highest AUC (0.94) implicative of the highest accuracy among the tests.

Detection of *M. avium* subsp. *paratuberculosis*-specific antibodies in experimentally infected calves. Using 95th and 99th percentile cutoffs, an initial *M. avium* subsp. *paratuberculosis*-specific IgG-based reactivity was detected by the FC assay in three *M. avium* subsp. *paratuberculosis*-infected calves (E9, E10, and E12) in group I and one calf (E15) in group II until up to 12 weeks postinoculation (wpi) (Fig. 2). An initial IgG1-based reaction was detected in calves E9 and E15 for up to 7 wpi.
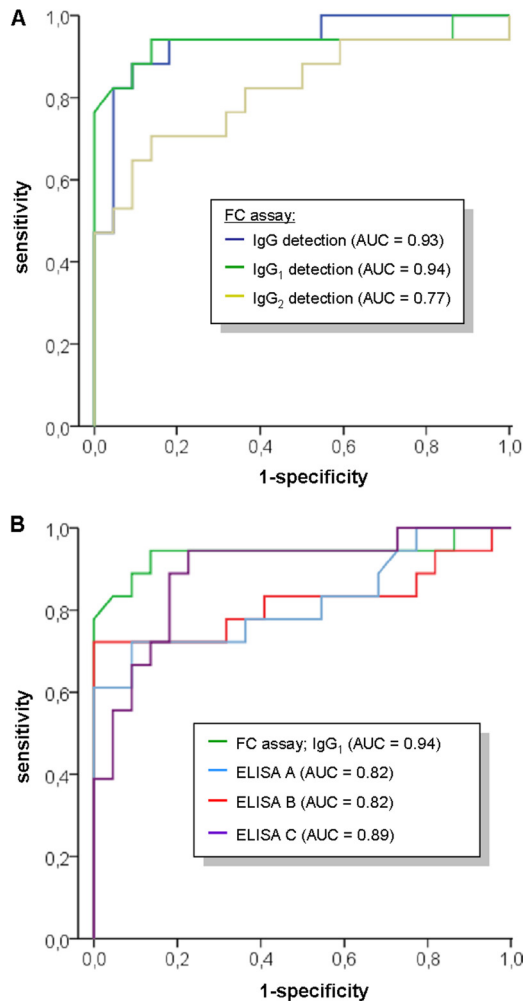
FIG 1 Results of the ROC curve analysis. Serum samples from 22 *M. avium* subsp. *paratuberculosis*-free and 18 *M. avium* subsp. *paratuberculosis*-shedding cows aged over 24 months were analyzed by FC assay and by three commercial ELISAs. The false-positive rate ("1-specificity" on the *x* axis) is plotted against the true-positive rate ("sensitivity" on the *y* axis) for every possible cutoff. Representative ROC curves were obtained from the results of the 40 serum samples when analyzed (A) by the FC assay considering immunoglobulin classes or subclasses IgG, IgG1, and IgG2 and (B) by three commercial ELISAs. AUC, area under the curve; ROC, receiver operating characteristic.

Sera from calf E7 tested positive or inconclusive for *M. avium* subsp. *paratuberculosis*-specific IgG as early as at 20, 28, and 32 wpi. E7 and E8 underwent an enduring *M. avium* subsp. *paratuberculosis*-specific seroconversion (IgG) later on, beginning at 44 and 46 wpi, respectively. This seroconversion could also be detected after 50 and 46 wpi when quantifying IgG1 in sera of calves E7 and E8, respectively. All calves in group II but one tested negative (IgG and IgG1) throughout the study. Serum taken from calf E13 at 44 wpi tested inconclusive (IgG).

An *M. avium* subsp. *paratuberculosis*-specific IgG2 response was detected in neither of the *M. avium* subsp. *paratuberculosis*-infected calves of group I. Three calves (E8, E10, and E12) had inconclusive results for IgM at 44, 36, and 40 wpi, respectively (data not shown). Based on these results, *M. avium* subsp. *paratuberculosis*-specific IgG2 and IgM responses were not assayed in *M. avium* subsp. *paratuberculosis*-infected calves of group II.

When serum samples were tested by ELISA B, the first sample from calf E9 tested positive and the last two samples (50 and 52 wpi) from calf E7 gave inconclusive results (with the cutoff prescribed by the manufacturer), while all other samples tested negative (Fig. 2). When applying the 95th and 99th percentile cutoffs instead, the first two samples and the sample taken at 54 wpi from calves E9 and E11, respectively, tested positive. *M. avium* subsp. *paratuberculosis*-specific seroconversion was detected by ELISA B in calf E7 after 44 wpi only.

Detection of *M. avium* subsp. *paratuberculosis*-specific antibodies in naturally exposed calves. To examine whether antibodies can be detected by the FC assay in calves that had been exposed to *M. avium* subsp. *paratuberculosis* in the field, serum samples from 32 calves in eight herds with a history of paratuberculosis and serum samples from 15 calves in three *M. avium* subsp. *paratuberculosis*-free herds were taken at days 100 ± 10 and 200 ± 30 postnatum (dpn). Mean *M. avium* subsp. *paratuberculosis*-specific IgG and IgG1 S/P ratios of calves in the paratuberculosis herd at both sampling points were not significantly higher than those of calves in *M. avium* subsp. *paratuberculosis*-negative herds (Fig. 3). Moreover, no significant increase of the average *M. avium* subsp. *paratuberculosis*-specific antibodies could be detected between the first and second samplings in the paratuberculosis herds.

All *M. avium* subsp. *paratuberculosis*-free calves tested IgG and IgG1 negative, while two calves in the paratuberculosis herd had a positive or inconclusive IgG result at 100 ± 10 dpn. At 200 ± 30 dpn, two other calves in the paratuberculosis herd had inconclusive results. Sera of none of the calves had positive *M. avium* subsp. *paratuberculosis*-specific IgG1 S/P ratios.

Dynamics of *M. avium* subsp. *paratuberculosis*-specific maternal antibodies in calves. All calves from FC assay-negative dams proved negative for *M. avium* subsp. *paratuberculosis*-specific antibodies by the FC assay (95th and 99th percentile cutoffs) during the first 6 months of life (data not shown). *M. avium* subsp. *paratuberculosis*-specific IgG and IgG1 S/P ratios were detected at least until 20 dpn in sera of all five calves (M1 to M5) from FC assay-positive dams. S/P ratios remained positive until 121 dpn and 50 dpn for IgG and IgG1, respectively (Fig. 4).

Calves from FC assay-negative dams tested negative by ELISA B (cutoff values prescribed by the manufacturer) (data not shown). Only two calves (M2 and M4) from FC assay-positive dams tested positive or inconclusive based on ELISA B until 63 and 104 dpn, respectively (Fig. 4).

DISCUSSION

A reasonable program to control paratuberculosis in dairy herds consists of strict prohibition of colostrum feeding from infected dams and subsequently effective prevention of calves from being infected with *M. avium* subsp. *paratuberculosis* via the fecal-oral route. The latter can only be achieved by stringent hygienic measures, including removal of any *M. avium* subsp. *paratuberculosis*-infected herd mates (calves and heifers), ideally before they start shedding the pathogen. As intrauterine infections also occur (17), diagnostic tests that allow for detection of *M. avium* subsp. *paratuberculosis* infections within the first months of life are imperative for the stepwise rehabilitation of a paratuberculosis herd when the ultimate goal is defined as eradication of *M. avium* subsp. *paratuberculosis* from that herd. Currently, neither accurate diagnostic methods for herd screening nor tests for early detection of *M. avium* subsp. *paratuberculosis* infections in calves are
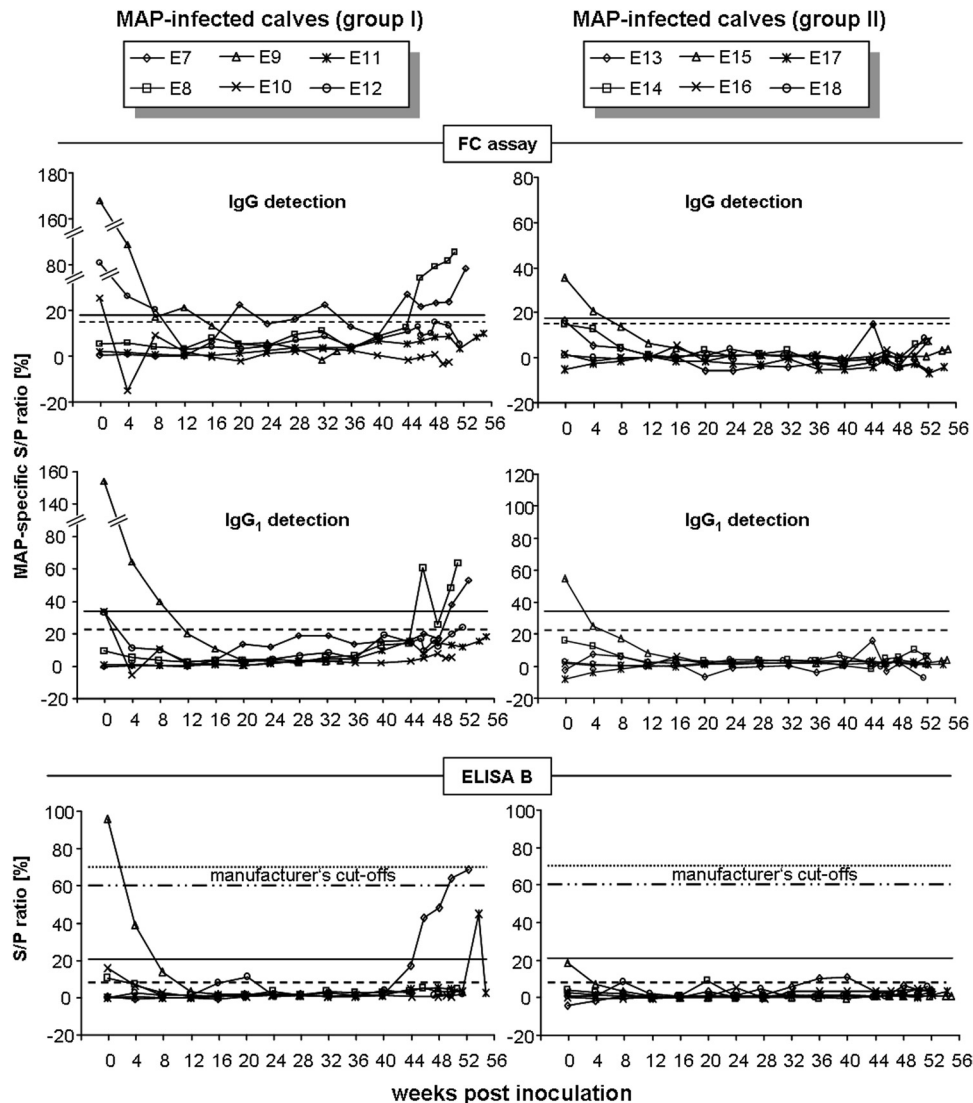
FIG 2 Dynamics of *M. avium* subsp. *paratuberculosis*-specific S/P ratios in experimentally infected calves. The curves represent the *M. avium* subsp. *paratuberculosis*-specific S/P ratios of 12 experimentally infected calves ("MAP-infected calves" in groups I [left] and II [right]) over a period of 55 weeks postinoculation (wpi). Serum samples were analyzed by FC assay (IgG and IgG1 detection, respectively) and by ELISA B. Cutoff values (horizontal lines) were defined as the 95th (dashed lines) and the 99th (solid lines) percentiles of all values obtained from six mock-infected control calves.

available. Compared to fecal culture and PCR tests, serological methods are inexpensive, rapid, and easy to perform. Even though identification of carrier individuals by serology is difficult for mycobacterial infections in general, we stepped out to improve the specificity and sensitivity of a novel FC-based serological assay introduced by Eda et al. (5). In this assay, using intact *M. avium* subsp. *paratuberculosis* bacteria as the test antigen, a full repertoire of unmodified *M. avium* subsp. *paratuberculosis* surface antigens is available for recognition by antibodies that may be supportive for the sensitivity of the assay. However, common epitopes of mycobacteria may result in cross-reactions and false-positive results (18). Initial studies with serum samples from adult cattle (data not shown) revealed that preabsorption with *M. phlei* antigen led to an increase in the specificity of IgG1 detection from 72.7% to 86.4% (at 94.4% sensitivity). Harvesting of the bacteria at the mid-log phase resulted in a better discrimination between

positive- and negative-control sera than using bacteria from the early log phase or the stationary phase (data not shown). The latter corroborates observations by Shin et al. (19) that serum samples from *M. avium* subsp. *paratuberculosis*-infected animals better recognize filtrates of mid-log-phase *M. avium* subsp. *paratuberculosis* cultures than filtrates of stationary-phase cultures. Recent studies also discovered that the proteome of *M. avium* subsp. *paratuberculosis* differs between bacteria propagated *in vitro* and bacteria obtained from the intestinal tissues of diseased animals (20, 21), implying that *in vitro*-grown bacteria may not express the full range of antigens to which infected animals are exposed. Further systematic studies on the correlation of growth conditions and serum reactivity may help to further improve the FC assay.

Comparison of the sensitivities and specificities of serological assays determined in different studies is difficult if a different standard is used to define *M. avium* subsp. *paratuberculosis*-positive
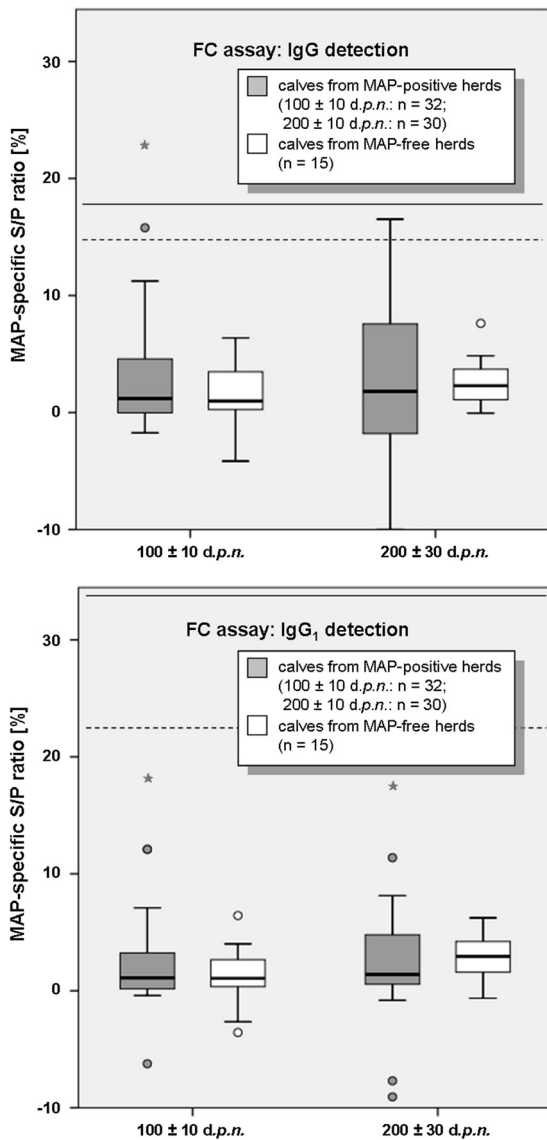
FIG 3 Distribution of *M. avium* subsp. *paratuberculosis* (MAP)-specific IgG and IgG1 S/P ratios in calves from *M. avium* subsp. *paratuberculosis*-positive herds. *M. avium* subsp. *paratuberculosis*-specific IgG (upper diagram) and IgG1 (lower diagram) S/P ratios from 32 and 30 calves, respectively, in eight herds with confirmed cases of paratuberculosis (gray box) and 15 calves from three *M. avium* subsp. *paratuberculosis*-free herds (white box) are depicted. Prior to statistical analysis (SPSS Statistic 17.0; SPSS Inc., Chicago, IL), a logarithmic transformation of the data was performed as they were not normally distributed. Student's *t* test was used to determine whether mean *M. avium* subsp. *paratuberculosis*-specific S/P ratios of both groups were significantly different from each other. For definition of cutoff values (horizontal lines), see the legend to Fig. 2. Circles indicate extremes, and stars indicate outliers.

FIG 4 Dynamics of *M. avium* subsp. *paratuberculosis*-specific (MAP) S/P ratios in calves from seropositive dams. *M. avium* subsp. *paratuberculosis*-specific S/P ratios of five calves from seropositive dams were determined by FC assay (IgG and IgG1 detection, respectively) and by ELISA B over a period of 250 dpn. For definition of cutoff values (horizontal lines), see the legend to Fig. 2.

and -negative cattle, if animals are sampled at different stages of infection, and if different numbers of animals are included. Nevertheless, data obtained in our study for ELISAs B (Pourquier ELISA Paratuberculosis) and C (Svanovir Para-Tb ELISA) compare well with data from other studies. Leroy et al. (22) tested ELISA B using 18 fecal culture-positive and 48 fecal culture-negative cows and found a sensitivity of 72% at a specificity of 92%. McKenna et al. (23) determined a sensitivity of 27.7% and specificity of 90.1% for ELISA C when using 36 fecal culture-positive
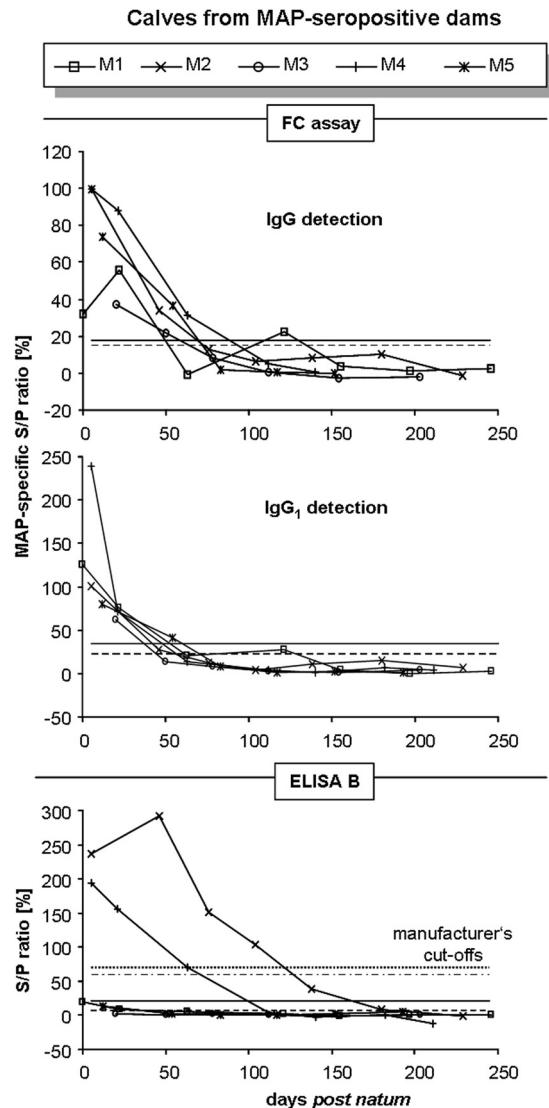
and 834 fecal culture-negative samples. Köhler et al. (24) classified dairy cattle on the basis of lesions characteristic of paratuberculosis and identified 286 healthy animals and 110 animals with paratuberculosis. With serum samples from these animals, ELISA B achieved 53.6% sensitivity and 99.3% specificity, while ELISA C was 70.0% sensitive and 76.6% specific.

When we assessed *M. avium* subsp. *paratuberculosis*-specific IgG1 in sera from adult cattle with confirmed fecal *M. avium* subsp. *paratuberculosis* shedding, the FC assay exhibited a considerably higher sensitivity of 78% at 100% specificity than commercial ELISAs with sensitivities ranging from 39% to 72%. For a more exact determination of the performance of the FC assay, a larger number of *M. avium* subsp. *paratuberculosis*-positive and -negative cows would have to be analyzed. However, our results are in line with the findings by Eda et al. (5), suggesting that the FC

assay may be more sensitive than ELISA B, which, e.g., is currently used to monitor for *M. avium* subsp. *paratuberculosis* infections in German dairy herds.

To define the earliest time point of detection, calves from *M. avium* subsp. *paratuberculosis*-free herds were experimentally infected and sampled over time. To our knowledge, 11 studies have been published since 1990 on the humoral immune response in calves experimentally infected with *M. avium* subsp. *paratuberculosis*. Challenge models varied mainly by strain, preparation of inoculum, route, dose and frequency of challenge, and age at challenge. Seven of these studies failed to detect a *M. avium* subsp. *paratuberculosis*-specific humoral immune response during the first year postinfection. In one experimental study, in which calves were challenged orally with gut mucosal tissue, one calf developed a high and persistent antibody titer starting at 9 months after inoculation (25). Three studies used identical serum samples from calves which underwent intratonsillar challenge with a total dose of 1.6×10^7 (5, 10, 26). Using a lipoarabinomannan-based ELISA, Waters et al. (10) detected *M. avium* subsp. *paratuberculosis*-specific antibodies in each of these three calves as early as 134 dpi and by Western blotting within the first 2 weeks of infection. Bannantine et al. (26) showed that antibodies against several *M. avium* subsp. *paratuberculosis* proteins can be detected in sera from two of the three calves as early as 70 dpi. The FC method established by Eda et al. (5) unveiled an *M. avium* subsp. *paratuberculosis*-specific seroconversion as early as 170 dpi. In the study presented here, the earliest time point at which an *M. avium* subsp. *paratuberculosis*-specific seroconversion could be detected by the FC assay was 44 wpi, corresponding to 307 dpi. Although we used a higher number of *M. avium* subsp. *paratuberculosis* strain K10 bacteria (1×10^9 CFU) as the challenge dose, the modified FC assay detected an *M. avium* subsp. *paratuberculosis*-specific seroconversion between 137 and 300 days later than the three studies using sera from the intratonsillarly infected calves. A possible reason for this delayed humoral immune response is the challenge route. Conceivably, the direct application of *M. avium* subsp. *paratuberculosis* in tonsillar crypts (i.e., close to one of the primary induction sites of immune responses) has resulted in a more efficient and earlier induction of a humoral immune response than a strict rumen/enteral inoculation. Still being somewhat artificial, our challenge model more closely resembled natural infections, and it is reasonable to consider that the kinetics of the immune responses better mimics the lapse after natural infection.

The aim of this study was to identify immune responses to *M. avium* subsp. *paratuberculosis* as early as possible in order to allow identification of *M. avium* subsp. *paratuberculosis*-infected calves ideally even before they had raised significant costs for husbandry. We followed a holistic approach and also aimed at detecting *M. avium* subsp. *paratuberculosis*-specific cell-mediated immune responses in the animals. To this end, we sampled calves on farms with or without known history of paratuberculosis and laboratory-confirmed *M. avium* subsp. *paratuberculosis* status as early as at about 100 and 200 dpn. Average FC assay S/P ratios in serum samples from calves at this age after natural exposure to *M. avium* subsp. *paratuberculosis* did not differ significantly from S/P ratios of calves unlikely to have had previous contact with *M. avium* subsp. *paratuberculosis*. Given the unknown time point of *M. avium* subsp. *paratuberculosis* infection in the setting of natural transmission in a herd, sampling even at 200 dpn might have been too early, therefore, to detect any seroconversion. In a separate

part of the study, animals were sampled at the age of 7 to 12 months and monitored by regular sampling for almost 2 years (data not shown). At the ages of 17 to 18 and 21 to 22 months, animals from a paratuberculosis-infected herd had significantly elevated S/P ratios when tested by the FC assay (IgG) but significantly lower S/P ratios when tested by ELISA B compared to *M. avium* subsp. *paratuberculosis*-free animals. Even though we were not able to prove the advantage of the improved FC assay for serological testing of young calves, the FC assay may be able to detect *M. avium* subsp. *paratuberculosis*-infected cattle at lower ages than the currently available indirect tests. Further studies will be needed, however, to substantiate this assumption.

Implementation and optimization of the FC assay in our lab required the usage of defined serum samples. As the gold standard to diagnose *M. avium* subsp. *paratuberculosis* infection of cattle is fecal culture, standard serum samples can only be obtained from animals that, if infected, are likely to shed *M. avium* subsp. *paratuberculosis* bacteria in sufficiently high numbers. This automatically results in the necessity to use samples from adult cattle. By applying the FC method to these samples, detection of *M. avium* subsp. *paratuberculosis*-specific IgG1 proved to be the most sensitive and specific approach. *M. avium* subsp. *paratuberculosis*-specific IgG2 was detected in 8 of the 18 fecal culture-positive cows only. These findings may be taken as evidence that IgG1 is the predominant immunoglobulin subclass after the onset of bacterial shedding when the animals have shifted from a T_h1- to a T_h2-biased immune response. Koets et al. (14) found both elevated IgG1 and IgG2 concentrations in sera of asymptomatic shedders compared to sera of animals in earlier or later stages of infection. Rather than being a general effect, the authors found that the increase in the respective Ig subclass was dependent on the antigen used. Therefore, conclusions on the relative contribution of IgG subclasses to the humoral immune response against *M. avium* subsp. *paratuberculosis* in cattle need to be drawn with care unless the assays are calibrated to immunoglobulin standards with known concentrations.

To the best of our knowledge, this is the first study examining the dynamics of Ig subclasses in *M. avium* subsp. *paratuberculosis*-infected calves. Given the predominance of T_h1 immune responses in the early phase of an *M. avium* subsp. *paratuberculosis* infection, we hypothesized that *M. avium* subsp. *paratuberculosis*-infected calves produce IgG2 rather than IgG1. However, an *M. avium* subsp. *paratuberculosis*-specific seroconversion regarding IgG1 was detected in two calves (E7 and E8) at 50 and 46 wpi, respectively, while IgG2 or IgM responses were undetectable in the *M. avium* subsp. *paratuberculosis*-infected calves. It remains an open question whether *M. avium* subsp. *paratuberculosis*-specific IgG2 and IgM were not produced in experimentally infected calves at all or whether levels of these antibodies only remained below the detection limit. Because infected calves could not be monitored until fecal *M. avium* subsp. *paratuberculosis* shedding was confirmed, the relative contribution of Ig subclasses to the specific immune response of cattle during the entire course of an *M. avium* subsp. *paratuberculosis* infection and the time of appearance of IgG2 could not be readily assessed.

M. avium subsp. *paratuberculosis*-specific antibodies were detected via FC assay in sera of all five calves from *M. avium* subsp. *paratuberculosis*-seropositive dams after colostrum ingestion but in none of the sera of calves from seronegative dams. Three experimentally infected calves also showed an initial *M. avium* subsp.

paratuberculosis-specific IgG and IgG1 responses at 0 to 12 wpi. The first sample of one of these calves also tested positive in the ELISA B assay. Calves for the experimental infection had been purchased from *M. avium* subsp. *paratuberculosis*-free herds, selected according to negative serological, PCR-based, and cultural tests of all animals aged over 24 months. The status of each dam was confirmed by collecting serum and fecal samples between 4 and 7 days after parturition. All fecal samples tested negative by culture and PCR and serum samples tested negative by ELISA B as well as by the FC assay. An explanation for the presence of *M. avium* subsp. *paratuberculosis*-specific antibodies in newborns could be the presence of "natural" antibodies with low avidity and low specificity. B1 cells are an important source of "natural" IgM, IgA, and IgG in humans and mice (27, 28). B1 cells differ from B2 cells in that they are generated predominately during fetal and neonatal development (29, 30). Studies performed in germfree, antigen-free, and maternal antibody-deprived animals showed that small amounts of "natural" antibodies are produced without any known cause of stimulation, presenting the first line of anti-infectious resistance (31). "Natural" antibodies are characterized by polyspecificity, enabling them to react with a broad spectrum of antigens, and thus they may interfere with serological testing of newborn animals' serum samples. Osterstock et al. (32) showed that false-positive serological reactions may also occur during use of commercially available ELISAs for paratuberculosis in calves experimentally exposed to environmental mycobacteria. Roussel et al. (18) could correlate a higher rate of false-positive ELISA results in beef herds with the amount of environmental mycobacteria cultured from feces. We introduced a preabsorption step with *M. phlei* in the FC assay protocol and normalized the reactivity to the reactivity of the serum to *M. avium* subsp. *avium* in order to minimize the effect of such cross-reactions. However, we did not determine to what extent the FC assay in the modifications evaluated herein can readily discriminate between *M. avium* subsp. *paratuberculosis*- and *M. avium* subsp. *avium*-induced humoral immune responses.

Taking cutoff values defined by calculating the 95th and 99th percentiles of all values obtained from six control calves of the experimental study, *M. avium* subsp. *paratuberculosis*-specific antibodies could be detected up to 121 dpn via the FC assay and up to 138 dpn via ELISA B. Still, the FC assay may be more sensitive in detecting maternal antibodies within the first weeks of life as it detected *M. avium* subsp. *paratuberculosis*-specific antibodies in all five calves from positive dams, while ELISA B identified only two calves. By definition, the detection of these antibodies does not reliably indicate whether a calf truly is infected. However, sanitizing efforts regularly involve hygiene measures, including prohibition of feeding colostrum, considered a principal source of infection, from infected cows to their offspring. A calf fed such colostrum must therefore be regarded as potentially infected. Irrespective of its true infection status, positive FC assay S/P ratios to *M. avium* subsp. *paratuberculosis* bacteria in the first weeks after birth strongly argue in favor of not introducing this calf into a herd at a farm participating in an *M. avium* subsp. *paratuberculosis* eradication program.

Conclusion. Even with the optimized FC assay, *M. avium* subsp. *paratuberculosis*-specific antibodies could only occasionally be detected in infected calves, indicating that the failure to detect such antibodies reflects the distinct immunobiology of *M. avium* subsp. *paratuberculosis* infection rather than methodolog-

ical constraints. Consequently, a serological approach may not be sensible enough for screening calves for *M. avium* subsp. *paratuberculosis* infections. Nevertheless, the FC assay with the modifications described and evaluated herein presented more sensitivity in the different applications tested in our study while retaining considerable specificity. It might thus be highly useful under certain conditions— e.g., experimental studies to understand the dynamics of humoral and cell-mediated immune responses. Implementation of this assay as an ancillary test also represents an interesting option in a setting when identification of calves which have had contact with *M. avium* subsp. *paratuberculosis* is of outmost importance, e.g., when introducing bull calves into semen collection centers or if an *M. avium* subsp. *paratuberculosis*-free herd is to be established after sanitizing efforts.

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