Simultaneous Detection of Antibodies against Apx Toxins ApxI, ApxII, ApxIII, and ApxIV in Pigs with Known and Unknown *Actinobacillus pleuropneumoniae* Exposure Using a Multiplexing Liquid Array Platform

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Surveillance for the presence of *Actinobacillus pleuropneumoniae* infection in a population plays a central role in controlling the disease. In this study, a 4-plex fluorescent microbead-based immunoassay (FMIA), developed for the simultaneous detection of IgG antibodies to repeat-in-toxin (RTX) toxins (ApxI, ApxII, ApxIII, and ApxIV) of *A. pleuropneumoniae*, was evaluated using (i) blood serum samples from pigs experimentally infected with each of the 15 known *A. pleuropneumoniae* serovars or with *Actinobacillus suis*, (ii) blood serum samples from pigs vaccinated with a bacterin containing *A. pleuropneumoniae* serovar 1, 3, 5, or 7, and (iii) blood serum samples from pigs with an unknown *A. pleuropneumoniae* exposure status. The results were compared to those obtained in a previous study where a dual-plate complement fixation test (CFT) and three commercially available enzyme-linked immunosorbent assays (ELISAs) were conducted on the same sample set. On samples from experimentally infected pigs, the 4-plex FMIA detected specific seroconversion to Apx toxins as early as 7 days postinfection in a total of 29 pigs inoculated with 14 of the 15 *A. pleuropneumoniae* serovars. Seroconversion to ApxII and ApxIII was detected by FMIA in pigs inoculated with *A. suis*. The vaccinated pigs showed poor humoral responses against ApxI, ApxII, ApxIII, and ApxIV. In the field samples, the humoral response to ApxIV and the *A. pleuropneumoniae* seroprevalence increased with age. This novel FMIA (with a sensitivity of 82.7% and a specificity of 100% for the anti-ApxIV antibody) was found to be more sensitive and accurate than current tests (sensitivities, 9.5 to 56%; specificity, 100%) and is potentially an improved tool for the surveillance of disease and for monitoring vaccination compliance.

*Actinobacillus pleuropneumoniae* is the causative agent of pleuropneumonia, a highly contagious disease in pigs that is responsible for substantial economic losses in global swine production systems (1). *A. pleuropneumoniae* can infect pigs of all ages, but clinical disease is mainly seen in growing pigs >12 weeks of age (2, 3). The disease is transmitted by the aerosol route or by direct contact and is often highly contagious. To date, 15 serovars of *A. pleuropneumoniae* have been described, varying in virulence and pathogenicity (4). Together with capsular polysaccharides and murein lipopolysaccharides, the extent of the virulence of the serovars is mainly determined by four different proteinaceous cytotoxins, ApxI, ApxII, ApxIII, and ApxIV, which belong to the pore-forming repeat-in-toxin (RTX) toxin family (5). The potential role of outer membrane proteins as virulence factors remains to be elucidated (6). Although ApxI, ApxII, and ApxIII are produced individually or in different combinations by different serovars of *A. pleuropneumoniae*, they are also found in pigs that are free of *A. pleuropneumoniae* as a result of infection with other less pathogenic Actinobacillus species, such as *Actinobacillus rossi* (which contains the apxI and apxII genes) and *A. suis* (which contains the apxII and apxIII genes) (7), and potentially in pigs infected with *Escherichia coli* or *Pasteurella* spp. (8, 9). In contrast, the ApxIV toxin has been found to be expressed in pigs infected with *A. pleuropneumoniae*, and it has been implicated as a marker to confirm *A. pleuropneumoniae* infection (10). However, ApxIV is not expressed under *in vitro* conditions (11).

The virulence patterns of different *A. pleuropneumoniae* serovars are associated with the exotoxins they express (12). ApxI is strongly hemolytic and cytotoxic and is expressed by the most virulent serovars, 1, 5, 9, 10, 11, and 14. ApxII is moderately hemolytic and cytotoxic and is secreted by all serovars except serovars 10 and 14. The strongly cytotoxic and nonhemolytic ApxIII is produced by serovars 2, 3, 4, 6, 8, and 15 (5, 12–15). Apx toxins are highly immunogenic and induce a strong antibody response following *A. pleuropneumoniae* infection (12). Antibodies against Apx toxins have been demonstrated in convalescent pigs using neutralization assays (16) and an indirect enzyme-linked immunosorbent assay (ELISA) (17).

Pigs that survive acute *A. pleuropneumoniae* infection and subclinically affected pigs develop a protective immunity and often continue to be infected carriers and sources of infection for other pigs, which may result in recurring disease outbreaks (4, 18). The identification of chronically or subclinically infected pigs, as well as the determination of the immune status on both a herd and an individual pig level, are important for the control and maintenance of populations that are free of the disease (1). To achieve...
this aim, different serological tests have been developed to detect antibodies against *A. pleuropneumoniae*. The complement fixation test (CFT) and various ELISAs have been the tests most extensively used for epidemiological surveillance and herd certification. The CFT (19), currently required for importation of live animals into the People’s Republic of China, is laborious, time-consuming, and expensive to use. Among the different ELISAs, those targeting ApxIV toxin to quantify circulating levels of the antibody to *A. pleuropneumoniae* (20), those targeting ApxI, ApxII, or ApxIII for the detection of specific antibodies to Apx exotoxins (21), those targeting capsular polysaccharides of *A. pleuropneumoniae* to identify antibodies against groups of its serovars (22), and serovar-specific ELISAs based on long-chain lipopolysaccharides (23) have been widely used and offer better sensitivities and specificities than the CFT. It has also been demonstrated that ELISAs utilizing different antigens or that are performed under different assay conditions may have conflicting results and cross-reactions between *A. pleuropneumoniae* serovars and other bacterial species, and this may be problematic (2, 3). The test specificity greatly varies depending on the serologic assay performed.

New sensitive immunological diagnostic tests, such as the chemiluminescence immunoassay (CLIA) (24) or the magnetic bead-based enzymatic spectrally fluorometric assay (25), have been recently introduced for the detection of single specific antibodies against the ApxIV toxin of *A. pleuropneumoniae*. However, these novel approaches, similar to ELISAs, are limited to measuring a single biomarker in a sample at a time. The relatively recent development of spectrally distinguishable fluorescent beads by Luminox Corp. (Austin, TX) (26) has resulted in the widespread use of antigen-coupled beads for simultaneous serological detection of antibodies to multiple antigen targets in a single assay using flow cytometry (27). Besides the multiplexing ability, the advantages of that platform, compared to conventional tests, such as the ELISA, is the enhanced analytical sensitivity and greater dynamic quantification range (28). Thus, smaller sample volumes are required and the procedure reduces the time and cost of the analysis (28). Despite these advantages, multiplex assays are rarely used for clinical diagnostic purposes in animals.

In this study, for the first time, a 4-plex fluorescent microbead-based immunoassay (FMA) for the simultaneous detection of blood serum antibody responses to *A. pleuropneumoniae* RTX toxins ApxI, ApxII, ApxIII, and ApxIV was developed and evaluated. Furthermore, this FMA was used to characterize the antigen antibody profile in blood serum samples obtained from experimentally infected pigs or from vaccinated pigs (known exposure) and from pigs kept under field conditions (unknown exposure), and its performance was compared against the currently available ELISAs and the CFT.

**Materials and Methods**

**Ethics statement.** The experimental protocol was approved by the Iowa State University Institutional Animal Care and Use Committee and by the Iowa State University Institutional Biosafety Committee. The field samples utilized were arbitrarily selected and originated from pig case submissions to the Iowa State University Veterinary Diagnostic Laboratory (ISU-VDL) for diagnostic workup. The sample collection and submission were unrelated to and not part of this study.

**Blood serum samples.** (i) Experimental samples of known *A. pleuropneumoniae* exposure. Experimental blood serum samples were used from a previous challenge study, the details of which have been described (3). All the original samples, including those that were excluded in the previous study due to the lack of any detectable antibodies, were tested by the novel 4-plex FMA. Briefly, 42 2- to 8-week-old pigs, obtained from a herd confirmed to be free of *A. pleuropneumoniae*, were assigned into challenged (pigs at ±5 weeks of age) or vaccinated (young pigs) groups. Among the challenged pigs, 34 animals were inoculated (2 to 4 pigs for each serovar) with *A. pleuropneumoniae* strains corresponding to serovars 1 to 15 or *A. suis*. In addition, eight pigs were vaccinated with in-house-produced bacterins based on serovar 1, 3, 5, or 7. Blood serum samples were collected at weekly intervals from the time of inoculation or vaccination until day 28 (3). Pigs infected with serovar 13 never seroconverted and were excluded from the study. The pigs used for evaluation were experimentally infected with *A. pleuropneumoniae* serovars 1 (n = 2), 2 (n = 2), 3 (n = 2), 4 (n = 4), 5 (n = 2), 6 (n = 2), 7 (n = 2), 8 (n = 2), 9 (n = 2), 10 (n = 2), 11 (n = 2), 12 (n = 2), 14 (n = 1), 15 (n = 2), or *A. suis* (n = 2).

(ii) Field samples from pigs with unknown *A. pleuropneumoniae* exposure. A total of 90 randomly selected blood serum samples collected from routine submissions to the Iowa State University Veterinary Diagnostic Laboratory were included. These serum samples corresponded to pigs from different production stages, including newborn pigs prior to their receiving colostral antibodies, sows with pigs born before (age n = 13), nursery pigs <60 days of age (n = 34), finisher pigs <25 weeks of age (n = 15), and adult pigs >25 weeks of age (n = 25). The pigs originated from 18 different farms in 5 U.S. states (CO, IL, IA, OK, and PA).

(iii) Sample prevalidation and reference tests. The samples were evaluated and validated in a previous report (3) by four different serological assays for the detection of antibodies against *A. pleuropneumoniae*. Specifically, a dual-plate CFT for serovars 1, 3, 5, and 7 (29, 30), a quad-plate indirect ELISA (ELISA-1; Swinecheck mix-APP 1-2-9-11, 3-6-8-15, and 4-5-7, and Swinecheck mix-APP 10-12 antibody test kits; Biovet, Inc., Quebec, Canada) composed of two kits with plates coated with lipopolysaccharide of certain serovars capable of detecting serovars 1 to 12, a single-plate indirect ELISA (ELISA-2; Cistvet Suis APP; Laboratories Hipra S.A., Girona, Spain) based on ApxI and surface transferring-binding protein 2 (Tbp2), and a single-plate indirect ELISA (ELISA-3; IDEXX APP-ApxIV antibody [Ab] test; IDEXX Laboratories, Inc., Westbrook, ME) based on a recombinant ApxIV antigen were used. The FMIA and ELISA-3 were directly compared, as both specifically targeted the detection of anti-ApxIV IgG antibodies (3).

Recombinant Apx toxin production and purification. (i) Recombinant fragments from toxins ApxI, ApxII, and ApxIII. Plasmids containing the *apxA* (*A. pleuropneumoniae* serovar 5) and *apxIIA* and *apxIIIA* (*A. pleuropneumoniae* serovar 2) genes were overexpressed in E. coli, as previously described (21). The recombinant His-tagged fusion toxins ApxI and ApxII were purified from extracts of *E. coli* BL21(DE3)pLysS (Rosetta cells) (Invitrogen, Carlsbad, CA) using nickel-nitrilotriacetic acid (Ni-NTA) chelate affinity chromatography, as described previously (21). The purification step of the original protocol (21) was modified for the recombinant ApxIII toxin. Briefly, a frozen pellet was obtained from a 100-ml culture of Rosetta cells containing the *apxIIIA* (615 bp) gene cloned in a pQE31 vector (QiAquick; Qiagen GmbH, Hilden, Germany) and overproducing ApxIII; this was thawed and resuspended in 1x lysis equilibration wash (LEW) lysis buffer (USB Corporation, Cleveland, OH) containing 8 M urea (Sigma-Aldrich, St. Louis, MO). The cells were lysed by sonication for 10 cycles of 20 s on ice using a Vibra-Cell sonicator (Sonics & Materials, Newtown, CT). The crude extract was centrifuged at 50,000 × g for 30 min at 4°C, and the supernatant was purified by Ni-NTA affinity chromatography using the PrepEase His-tagged protein purification kit according to the manufacturer’s instructions (USB Corporation). The apparent molecular weight of each polypeptide was determined by SDS-PAGE.

(ii) Recombinant fragment from toxin ApxIV. Genomic DNA from *A. pleuropneumoniae* serovar 3 (strain ATCC 27090) was extracted using the QIAamp DNA blood mini kit (Qiagen, Valencia, CA) according to the
Four-plex FMIA development. (i) Coupling of Apx recombinant toxins to magnetic microbeads. The covalent coupling of purified recombinant Apx toxins was performed following a previously reported method (32) with minor modifications based on a two-step carbodiimide procedure similar to that of a previously described assay (32). All incubations were done at room temperature (20 to 25°C) in the dark on a rotator shaker (set at 500 to 600 rpm). Briefly, beads sets carrying different recombinant toxins were sonicated, mixed by vortexing, and diluted in StabilGuard buffer (SurModics, Eden Prairie, MN) to a final concentration of 1.0 × 10⁶ beads per well (2,500 beads/well for each type). The blood serum samples were diluted 1:50 in assay buffer (0.1 M phosphate-buffered saline [PBS], 10% goat serum [Gibco, Life Technologies, Grand Island, NY], 0.05% Tween 20 [pH 7.2]) and mixed into each well with 50 μl of the bead suspension. The mixture was incubated for 30 min and washed three times with PBS containing 0.05% Tween 20 (PBST). Next, 50 μl of a 1:3,000 dilution of biotin-conjugated goat anti-swine IgG (Jackson ImmunoResearch, Inc., West Grove, PA) in assay buffer was added to each well and incubated for 30 min. Finally, after three washing steps, the beads were resuspended in 100 μl of assay buffer. The reporter fluorescence of the beads was determined by a dual-laser Bio-Rad Bio-Plex 200 instrument with the Bio-Plex Manager software version 6.0 (Bio-Rad) and expressed as the median fluorescent intensity (MFI) of ≥50 microspheres per set per well. Using the default settings in this study, the Bio-Rad Bio-Plex software gates all results to recognize double beads or aggregates of beads so they can be excluded or retested as applicable. A set of beads coupled at the same time was used for all samples to limit intercoupling variation. Uncoupled microspheres incubated with a positive sample (selected as standard) and coupled microspheres incubated with a positive control (high MFI) (ApxI, 11,180; ApxII, 12,132; ApxIII, 12,132; ApxIV, 7,167), a negative control (low MFI) (ApxI, 196; ApxII, 435; ApxIII, 457; ApxIV, 176), and a cutoff internal control sample (MFI around the cutoff value) (ApxI, 2,700; ApxII, 3,800; ApxIII, 3,400; ApxIV, 1,670), as well as coupled microspheres incubated with assay buffer in the absence of any sample (negative control or background), were included as controls in each run to monitor assay reproducibility and precision. The MFI data were corrected for background (Bkg) levels by subtracting the negative antigen signal from the positive antigen signal (MFI-Bkg).

(ii) Four-plex FMIA procedure. The FMIA was performed with a procedure similar to that of a previously described assay (32). All incubations were done at room temperature (20 to 25°C) in the dark on a rotating shaker (set at 500 to 600 rpm). Briefly, beads sets carrying different recombinant toxins were sonicated, mixed by vortexing, and diluted in StabilGuard buffer (SurModics, Eden Prairie, MN) to a final concentration of 1.0 × 10⁶ beads per well (2,500 beads/well for each type). The blood serum samples were diluted 1:50 in assay buffer (0.1 M phosphate-buffered saline [PBS], 10% goat serum [Gibco, Life Technologies, Grand Island, NY], 0.05% Tween 20 [pH 7.2]) and mixed into each well with 50 μl of the bead suspension. The mixture was incubated for 30 min and washed three times with PBS containing 0.05% Tween 20 (PBST). Next, 50 μl of a 1:3,000 dilution of biotin-conjugated goat anti-swine IgG (Jackson ImmunoResearch, Inc., West Grove, PA) in assay buffer was added to each well and the plate was incubated for 30 min. Next, 50 μl of streptavidin-phyceroerythrin (SA-PE) (Moss, Inc., Pasadena, MD) (2.5 μg/ml in assay buffer) was added to each well and incubated for 30 min. Finally, the fluorescent intensity (MFI) was optimized by selecting the conditions that produced the most consistent and reproducible results. A total of 12.5 μg of ApxI (50 μg/5 × 10⁶ beads), 7.7 μg of ApxII (30 μg/5 × 10⁶ beads), 12.6 μg of ApxIII (50 μg/5 × 10⁶ beads), and 19 μg of ApxIV (75 μg/5 × 10⁶ beads) were coupled to 1.25 × 10⁶ carboxylated fluorescent microbeads. The following bead regions were used for the coupling reaction: 26 (ApxI), 34 (ApxII), 52 (ApxIII), and 43 (ApxIV). Since different purification pathways with differing performances were used to purify each recombinant Apx toxin, and because of differences in the antigenic performances of individual toxins, the antigenic mass of each toxin coupled to the Luminex microspheres, as well as the coupling conditions, were optimized during the assay development. ApxI and ApxII, expressed as soluble proteins, were coupled using 2-(N-morpholino)ethanesulfonic acid (MES) (pH 5.03), while both ApxIII and ApxIV, expressed as insoluble aggregates (inclusion bodies in the pellet) and purified under denaturing conditions (8 M urea), were coupled using MES (pH 6.01). In addition to the coupled microspheres, one set of microspheres per region was prepared as a negative control by leaving out the capture antigens.

(iv) Assay procedure optimization. Specific recombinant ApxI, ApxII, ApxIII, and ApxIV toxins were first evaluated separately in a single-analyte xMAP setup by an analysis of specific positive and negative experimental serum controls, which were previously evaluated and validated by other complementary techniques, such as ELISA and a CFT (3). Different assay parameters, such as concentrations of the reagents, incubation temperatures and time, and buffer composition, were investigated. The toxins that worked properly in the single-analyte assay were sequentially added into the 4-plex assay. The absence of matrix effects, such as the interactions among antigens, nonimmune specific binding, and multiplexing cross-interference that may compromise assay results, were analyzed directly from changes in the selected controls or by the use of different combinations of the recombinant antigens. The design included the removal of one set of toxins (one of four microsphere subclasses) at a time from the 4-plex FMIA reaction mixture preparation.

To confirm the reproducibility of this method, intraassay (four strong-positive- and three weak-positive-control samples tested three times within an experiment) and interassay (two strong-positive samples and one weak-positive-control sample tested one time in three different experiments) precision were evaluated, and the mean, standard deviation (SD), and coefficient of variation (CV) were calculated for each repeated measurement (34). In addition, all experimentally derived samples and all field samples were analyzed in duplicate in two independent runs.

The positive, cutoff, and negative internal controls were prepared to monitor subsequent runs. The cutoff internal control represents a previously selected sample with a result within the cutoff. The cutoff internal control was used to calculate the antibody index of each particular sample based on the following formula: antibody index = sample MFI or MFI-Bkg/cutoff serum mean MFI or MFI-Bkg. Samples with an antibody index value of <0.9 were considered negative, samples with an antibody index value of ≥1.1 were considered positive, and samples with an antibody index value between 0.9 and 1.1 were considered suspect. Suspect samples were retested once. If the result was in the suspect range, these samples were reported as positive.

Data analysis. The sensitivity and specificity of the 4-plex FMIA to detect species-specific IgG antibodies against A. pleuropneumoniae were
was carried out to determine the sensitivity and specificity of the negative samples (data not shown). The differences among the assays were investigated by comparing the MFIs generated by the positive and negative cutoffs, which were chosen based on the maximized diagnostic sensitivity and specificity of the 4-plex FMIA relative to the other assays. The ability of the 4-plex FMIA to discriminate the positive and negative samples (sensitivity and specificity) was evaluated using receiver operator characteristic (ROC) curve analysis.

Statistical analysis. McNemar's test for pair-wise comparison was used to determine whether the proportions of positive samples were significantly different among the assays. The differences between the groups were considered significant if the P value was <0.05. A kappa index was calculated to determine the agreement of positive/negative results between ELISA-3 (IDEXX APP-ApxIV Ab test, IDEXX Laboratories, Inc., Westbrook, ME) and the ApxIV portion of the 4-plex Apx FMIA. The strength of agreement was considered poor if the kappa index was ≤0, slight if 0.01 to 0.2, fair if 0.21 to 0.4, moderate if 0.41 to 0.60, substantial for 0.61 to 0.80, and almost perfect if 0.81 to 1, as described previously (35). Statistical analyses were performed using SAS version 9.2 (SAS Institute, Inc., Cary, NC).

RESULTS

Optimization of the 4-plex FMIA. The assay precision was evaluated using specific experimental blood serum control samples. Prior to the 4-plex FMIA development, both intraassay and interassay variability were measured for each individual single-plex FMIA, generating intraassay and interassay CVs of ≤3% and ≤7.0%, respectively (Table 1) (34). Once the performance of each bead set was verified to be correct in a single-plex format, the interference and cross-reactivity between the different bead sets were investigated by comparing the MFIs generated by the positive- and negative-control sera in the single-plex assays with those generated by the 4-plex FMIA. These results were analyzed by linear regression, and correlation coefficients (R values) were determined (Table 1). As shown in Table 1, a good correlation between the single-plex and 4-plex FMIA was achieved for all Apx toxins, and no cross-reactivity (binding of antibody with the wrong protein or bead set) was observed between the bead sets. With the exception of slight fluctuations in the signal for some samples, the MFIs generated by the 4-plex FMIA were similar to those generated by the single-plex assay. In addition, the reproducibility of the 4-plex FMIA was evaluated by testing all the samples in duplicate in two independent assays (34), generating optimal values of <10% CV for positive samples and <15% CV for negative samples (data not shown).

Cutoff determination for the 4-plex FMIA. An ROC analysis was carried out to determine the sensitivity and specificity of the FMIA. The overall ROC optimized cutoff was calculated using the cumulative data from all experimental samples, thereby avoiding the loss of specificity in favor of an increase of the detection rate in the early stages postinfection. Since the toxins ApxI, ApxII, and ApxIII are also produced by some bacterial species other than A. pleuropneumoniae and because some pigs had detectable antibody levels to ApxII and ApxIII at the time of the experimental inoculation, the selected cutoffs were chosen to retain specificity even at the cost of sensitivity. The cumulative area under the ROC curve (AUC) indicated that the 4-plex Apx FMIA was 100% accurate. The optimal cutoff values determined by ROC analysis were determined to be a sample MFI value of 2,700 (range between 2,680 and 2,880) for ApxI, 2,800 (2,680 and 2,810) for ApxII, 3,400 (3,390 and 3,790) for ApxIII, and 1,670 (1,550 and 1,740) for ApxIV. For that cutoff, the diagnostic sensitivity was 68% for ApxI, 64.5% for ApxII, 98.2% for Apx III, and 82.7% for ApxIV, while the diagnostic specificity was 73.6% for ApxI, 0% for ApxII, 28.3% for ApxIII, and 100% for ApxIV.

Anti-Apx toxin antibody profile in experimental samples from pigs with known exposure to A. pleuropneumoniae. (i) Overall seroconversion and exclusion of pigs. Pigs that seroconverted to both ApxIV and any combination of the other three Apx toxins were considered positive. All the animals and serovars detected by any of the tests previously evaluated were positive for antibodies against one or more Apx toxins. In addition, the FMIA was able to detect anti-ApxIV antibodies in 7 pigs that had no detectable antibody response by any of the previously evaluated assays (Table 2). However, 2/2 pigs inoculated with serovar 13 and 1/2 pigs inoculated with serovar 14 were confirmed to be noninfected and thus were excluded from the study. The overall sensitivity obtained using the 4-plex FMIA is presented in Table 2. Except for serovars 3, 7, and 11, the ApxIV FMIA was more sensitive overall for species-specific A. pleuropneumoniae serodetection than any possible combination of the previously utilized tests. Specifically, seroconversion was detected against 14 different A. pleuropneumoniae serovars in a total of 29 experimentally infected pigs. In contrast, all the reference tests combined were able to detect 13 A. pleuropneumoniae serovars in a total of 22 pigs.

(ii) ApxI FMIA profile by serovar. Seroconversion to ApxI was detected for A. pleuropneumoniae serovars 1, 5, 9 to 11, and 14, for a total of 44 blood serum samples, and for A. suis in 8 serum samples. Among the samples from pigs that were infected experimentally with A. pleuropneumoniae, 30 of 44 samples expected to be positive showed seroconversion, while 19 samples expected to be negative were positive between 7 and 28 days postinfection (dpi), resulting in a sensitivity of 68.2% and a specificity of 73.6% (Table 2). None of the 31 samples collected before inoculation showed substantial levels of anti-ApxI IgG. A total of 14 pigs inoculated with one of nine different serovars (serovars 1, 3 to 5, and 8 to 12) seroconverted to ApxI between 7 and 21 dpi. No seroconversion to ApxI was detected in the pigs inoculated with A. pleuropneumoniae serovars 2, 6, 7, 14, and 15 (Table 2). The time until first detection of the antibody-positive pigs infected with a certain serovar ranged from day 7 (for serovars 1, 4, 5, and 9 to 12) to 21 (for serovar 3) (Table 3). Once a pig that tested positive had a detectable anti-ApxI IgG response, it continued to be positive for the subsequent bleeding days, except for one pig inoculated with serovar 5 and another pig inoculated with serovar 11 that both had detectable levels of anti-ApxI IgG by 7 dpi and subsequently became seronegative (Table 3). Pigs inoculated with serovars 10, 11,
TABLE 2 Detection rate of anti-Apx toxin antibody-positive pigs by the 4-plex fluorescent microbead-based immunoassay compared to previously utilized assays based on similar (ApxIV; ELISA-3) or other antigens (3) on serum samples collected at days 7, 14, 21, and 28 after experimental inoculation with various *Actinobacillus pleuropneumoniae* serovars or *Actinobacillus suis*.

| Serovar (no. of pigs) | Detection rate with previously used antibody detection assays | Detection rate with 4-plex FMIA by Apx toxin: |
|-----------------------|-----------------------------------------------------------|-------------------------------------------|
|                       | Dual-plate CFT  | Quad-plate ELISA | Combined detection rate |
|                       | 1-5  | 7 | 9-11 | 3 | 6-8-15 | 4 | 5-7 | 10-12 | ELISA-2 | ELISA-3 | (%) |
| A. pleuropneumoniae   |       |   |     |     |       |   |     |     |       |       |     |
| 1 (2)                 | 1/8b  | 0/8 | 1/8b | 0/8 | 0/8  | 0/8 | 1/8b | 2/8 (2)b | 100 (8/8) | 8/8b  | 8/8b  | 8/8b |
| 2 (2)                 | 1/8   | 0/8 | 1/8 (1)b | 0/8 | 0/8  | 0/8 | 1/8b | 0/8b  | 37.5 (3/8) | 0/8  | 8/8b  | 8/8b |
| 3 (2)                 | 0/8   | 0/8b | 0/8 | 7/8 (1)b | 0/8 | 0/8  | 0/8b | 0/8b  | 87.5 (7/8) | 2/8  | 3/8 (1)b | 8/8 (1)b |
| 4 (4)                 | 3/16  | 0/16 | 0/16 | 0/16 (b) | 0/16 | 2/16(b) | 1/16 (1)b | 18.7 (3/16) | 10/16 (1) | 12/16 (1)b | 16/16b  | 14/16 (3)b |
| 5 (2)                 | 0/8b  | 0/8 | 0/8 | 4/8 (4)b | 0/8 | 0/8b | 0/8b  | 50 (4/8) | 1/8b | 0/8b  | 0/8 |
| 6 (2)                 | 5/8   | 5/8b | 0/8 | 6/8b  | 0/8 | 0/8b | 1/8 (1)b | 75 (6/8) | 0/8  | 7/8b  | 7/8b |
| 7 (2)                 | 4/8b  | 0/8 | 8/8 (2)b | 0/8 | 0/8b | 0/8b  | 50 (4/8) | 0/8  | 4/8b  | 8/8 |
| 8 (2)                 | 0/8   | 3/8 | 0/8 | 4/8 (1)b | 0/8 | 0/8b | 1/8b  | 50 (4/8) | 3/8  | 4/8 (1)b | 8/8b |
| 9 (2)                 | 1/8b  | 0/8 | 8/8b  | 0/8 | 0/8b | 0/8b  | 100 (8/8) | 8/8b | 8/8b |
| 10 (2)                | 1/8b  | 0/8 | 0/8b | 2/8 (2)b | 0/8b | 3/8 (2)b | 62.5 (5/8) | 8/8b | 7/8b  | 3/8 |
| 11 (2)                | 2/8b  | 0/8 | 8/8 (2)b | 0/8b | 0/8b | 0/8b  | 100 (8/8) | 5/8b | 5/8b  | 6/8b |
| 12 (2)                | 0/8b  | 0/8 | 0/8 | 8/8 (1)b | 0/8b | 3/8b (3)b | 87.5 (7/8) | 3/8 | 7/8b  | 8/8 |
| 13 (1)                | 0/4   | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4  | 0/4  | 0/4 |
| 15 (2)                | 0/8b  | 0/8 | 0/8 | 4/8 (1)b | 0/8 | 0/8b | 0/8b  | 50 (4/8) | 0/8  | 8/8b |

A. suis (2)

| 0/8 | 0/8 | 0/8 | 0/8 | 0/8 | 0/8 | 0/8 | 0/8 |

| ApxI | ApxII | ApxIII | ApxIV |
|------|-------|--------|-------|
| 8/8b | 8/8b  | 8/8b   | 8/8b  |

*For the purpose of this study, the suspect samples as defined in the manufacturer’s instructions were considered positive. The number of samples out of the total for each assay within the suspect range for each serovar is given in parentheses after the prevalence. The values in boldface indicate that at least one positive animal was present.

b Serovar(s) that should be detected by the respective assays.

and showed the highest antibody responses against ApxI. No anti-ApxI IgG response was detected in pigs inoculated with A. suis (Table 2) or in any of the vaccinated pigs (data not shown).

(iii) ApxII FMIA profile by serovar. Seroconversion to ApxII was expected after inoculation with *Actinobacillus pleuropneumoniae* serovars 1 to 9, 11, 12, and 15, for a total of 104 blood serum samples, and for *A. suis* in 8 serum samples. Among the samples from pigs infected experimentally with *A. pleuropneumoniae*, 67 of the 104 samples expected to be positive showed seroconversion, while 12 samples were unexpectedly positive for anti-ApxII IgG between 7 and 28 dpi, resulting in a sensitivity of 64.4% and a specificity of 0% (Table 2). A total of 21 pigs inoculated with each of 12 different serovars (serovars 1 to 4, 6 to 12, and 14) seroconverted to ApxII between 7 and 14 dpi (Table 3). Contrary to our expectations, no

TABLE 3 Detection rates and seroconversion patterns to Apx toxins for *Actinobacillus pleuropneumoniae* and *Actinobacillus suis* on serum samples collected on days 7, 14, 21, and 28 after experimental inoculation as determined by a 4-plex fluorescent microbead-based immunoassay

| A. pleuropneumoniae serovar | No. detected/no. of total for each toxin by dpi: |
|-----------------------------|-----------------------------------------------|
| ApxI                        | ApxII                                       |
| 0       | 7        | 14       | 21       | 28 |
| 1        | 0/2       | 2/2       | 2/2       | 2/2  |
| 2        | 0/2       | 0/2       | 0/2       | 0/2  |
| 3        | 0/2       | 0/2       | 1/2       | 1/2  |
| 4        | 0/4       | 1/4       | 3/4       | 3/4  |
| 5        | 0/2       | 0/2       | 0/2       | 0/2  |
| 6        | 0/2       | 0/2       | 0/2       | 0/2  |
| 7        | 0/2       | 0/2       | 0/2       | 0/2  |
| 8        | 0/2       | 0/2       | 1/2       | 1/2  |
| 9        | 0/2       | 0/2       | 2/2       | 2/2  |
| 10       | 0/2       | 2/2       | 2/2       | 2/2  |
| 11       | 0/2       | 2/2       | 2/2       | 2/2  |
| 12       | 0/2       | 1/2       | 1/2       | 1/2  |
| 13       | 0/2       | 0/2       | 0/2       | 0/2  |
| 14       | 0/1       | 0/1       | 0/1       | 0/1  |
| A. suis  | 0/2       | 0/2       | 0/2       | 0/2  |

*Expected serological toxin pattern (47). 

b Pigs that were positive at the time of the inoculation.
seroconversion to ApxII was detected in any of the pigs inoculated with serovar 5 or 15. Among the 31 blood serum samples collected prior to *A. pleuropneumoniae* inoculation, one serovar 1 pig and one serovar 15 pig had detectable antibody levels to ApxII (slightly above cutoff limit). The pig inoculated with serovar 1 showed a strong humoral response to ApxII at 7 dpi, while in the serovar 15 pig, the anti-ApxII IgG levels fell below the detection limit by 14 dpi (Table 3). The time until detection of antibody-positive pigs infected with a certain serovar ranged from day 7 (for serovars 1, 2, 4, 6, 7, 9 to 12, and 14) to 14 (for serovars 3 and 8). In all cases, once a pig had a detectable anti-ApxII-IgG response after *A. pleuropneumoniae* inoculation, it continued to be positive on the subsequent bleeding days, with MFI peak values between 21 and 28 dpi (Table 3). Except for serovar 14, which had the lowest MFI values, the antibody levels were similar and independent of the serovar used in inoculation (data not shown). Pigs inoculated with *A. suis* (2/2) showed clear seroconversion against ApxII by 7 dpi and remained positive throughout the duration of the study (Table 3). Among the vaccinated pigs, only one pig vaccinated with serovar 7 seroconverted by day 7, and this pig decreased to a suspect level by day 28. Two pigs vaccinated with serovar 5 had a recognizable antibody response against ApxII.

(iv) **ApxIII FMIA profile by serovar.** Seroconversion to ApxIII was expected after inoculation with *A. pleuropneumoniae* serovars 2 to 4, 6, 8, and 15, for a total of 56 blood serum samples. Seroconversion to ApxIII has not been reported for *A. suis*. Among the samples from pigs experimentally infected with *A. pleuropneumoniae*, 55 of 56 samples expected to be positive showed seroconversion, while 47 samples were unexpectedly positive for ApxIII between 7 and 28 dpi, resulting in a sensitivity of 98.2% and a specificity of 28.3% (Table 2). A total of 22 pigs inoculated with one of 12 different serovars (serovars 1 to 4, 6 to 12, 14, and 15) seroconverted to ApxIII sometime between day 7 and 14 dpi (Table 2). In accordance with the established cutoff, neither of the two pigs inoculated with serovar 5 showed specific seroconversion against ApxIII after inoculation. However, among the 31 serum samples collected prior to infection, one pig inoculated with serovar 5 and one pig inoculated with serovar 7 had detectable levels of anti-ApxIII IgG, which remained stable and above the detection limit throughout the duration of the study (Table 3). The time until first detection of antibody-positive pigs infected with certain serovars ranged from day 7 (for serovars 1 to 4, 6 to 9, 11, 12, 14, and 15) to 14 (for serovar 10). Most of the antibody-positive pigs reached peak levels of anti-ApxIII IgG by days 7 to 14 after inoculation (Table 3). In all cases, once a pig seroconverted against ApxIII, it continued to be FMIA positive for the subsequent bleeding days (Table 3). The highest humoral response (MFI signal) against ApxIII was found in individual pigs inoculated with serovars 2, 4, and 6 (data not shown). All pigs inoculated with *A. suis* (2/2) showed a clear and very strong seroconversion to ApxIII by 7 dpi and remained positive for the duration of the study (Table 3). Among the vaccinated pigs, only one pig vaccinated with serovar 7 (1/2 pigs seroconverted on day 7) showed a detectable seroconversion against ApxIII. The other pig vaccinated with serovar 7 was positive at the time of vaccination; however, no increase in the antibody response to ApxIII was detected after vaccination.

(v) **ApxIV FMIA profile by serovar.** Among all the samples, 96 samples were considered seropositive and 20 were considered seronegative for ApxIV between days 7 and 28 after inoculation, resulting in a sensitivity of 82.7% and a specificity of 100% (Table 2). None of the 31 samples collected before inoculation had a detectable anti-ApxIV IgG level. Specific seroconversion against ApxIV was detected after inoculation with each one of the 14 serovars. The first detection of antibody-positive pigs infected with a certain serovar ranged from dpi 7 (for serovars 1 to 12) to 14 (for serovars 14 and 15). In all cases, once a pig seroconverted to ApxIV, it continued to be FMIA positive for the subsequent bleeding days (Table 3). The detection signal increased as the days post-exposure increased; most of the positive animals showed low-positive MFI values within the first 2 to 3 weeks after inoculation and reached peak levels by 28 dpi (Table 3). In all the cases, pigs that showed seroconversion to ApxIV after *A. pleuropneumoniae* inoculation were also seropositive to one or more other Apx toxins. Cross-reaction was not observed with *A. suis*. The FMIA and ELISA-3 showed only a slight agreement for anti-ApxIV antibody detection due to the very low detection rate for ELISA-3 (k = 0.097, P < 0.0001). Among the vaccinated pigs and contrary to ELISA-3, for which no seroconversion to ApxIV was detected in any vaccinated pig, one pig vaccinated with serovar 1 (1/2 pigs seroconverted on day 14), one pig vaccinated with serovar 3 (1/2 pigs seroconverted on day 21), and one pig vaccinated with serovar 7 (1/2 pigs slightly seroconversion on day 28) showed a small increase in anti-ApxIV-IgG response after vaccination.

**Apx toxin profiles in field samples from pigs with unknown *A. pleuropneumoniae* exposure.** The seroprevalence rates for individual anti-ApxI, anti-ApxII, anti-ApxIII, and anti-ApxIV IgG using the 4-plex FMIA on field samples obtained from pigs in different stages of production are presented in Fig. 1. Based on the recommended cutoff criteria, as with the reference test, anti-Apx antibodies were not detected in newborn pigs before colostrum uptake. In general, the serological profiles for antibodies against the non-species-specific toxins ApxI, ApxII, and ApxIII appeared to vary with age, characterized by high antibody levels in suckling pigs (64% to 100%), which gradually declined in nursery pigs (41% to 82%), and reached minimum levels in grow-finish pigs (27% to 47%) and maximum levels in adults (100%) (Fig. 1A). In contrast, for *A. pleuropneumoniae* species-specific serodetection, the seroprevalence of anti-ApxIV IgG increased with age, ranging from 18% (2/11) in sucking pigs to 100% (25/25) in adults (Fig. 1A). When the seroresponse against ApxI, ApxII, and ApxIII were analyzed separately for ApxIV-positive samples (n = 43) (Fig. 1B) and ApxIV-negative samples (n = 47) (Fig. 1C), it was found that for Apx IV-positive samples, Apx seroprevalence increased with age, while for ApxI- and ApxIII-positive samples, seroprevalence reached maximum levels in sucking and adult pigs. For the ApxIV-negative samples, the seroprevalences of ApxI, ApxII, and ApxIII antibodies decreased with age and were nonexistent in the adult pigs. Although the FMIA and the ELISA-3 were in moderate agreement (k = 0.46), mainly due to discrepancies in the late stages of production. For different stages of production, the agreement between FMIA and ELISA-3 was almost perfect for sucking pigs (k = 0.002), mainly due to discrepancies in the late stages of production. For different stages of production, the agreement between FMIA and ELISA-3 was perfect for sucking pigs (k = 0.001). The antibody detection patterns against different Apx toxins in field samples obtained by the 4-plex FMIA are presented in Table 4.
**TABLE 4** Anti-Apx toxin antibody patterns obtained by a 4-plex fluorescent microbead-based immunoassay in field samples from pigs of unknown *A. pleuropneumoniae* exposure collected from 18 U.S. farms

| Pig type/age               | No. of pigs | No. of Apx negatives |
|----------------------------|-------------|----------------------|
| Fetus/neonate*b            | 5           | 5                    |
| Suckling, 0–20 days        | 11          |                      |
| Nursery, 21–55 days        | 34          | 3                    |
| Grow-finish, 8–25 wk       | 15          | 6                    |
| Adult, >25 wk              | 25          |                      |

*No. of samples with *A. pleuropneumoniae* result by Apx type(s):*

| Positive | Negative |
|----------|----------|
| I + IV   | I + IV   |
| II + IV  | II + IV  |
| I + II + IV | I + II + IV |
| II + III + IV | II + III + IV |
| I + II + III + IV | I + II + III + IV |

In accordance with the established cutoff, pigs with significant antibody levels against both ApxIV and any combination of ApxI, ApxII, and/or ApxIII were considered to be *A. pleuropneumoniae* seropositive, while pigs with negative levels of anti-ApxIV antibodies were considered to be *A. pleuropneumoniae* seronegative.

*b Sample collection was prior to colostrum uptake.

**DISCUSSION**

Monitoring the *A. pleuropneumoniae* immune status of swine herds and the identification of “carriers” is very important to avoid the introduction of infected animals into naïve populations where disease caused by *A. pleuropneumoniae* can be devastating (1). Among the wide range of laboratory tests that can be used for the diagnosis of *A. pleuropneumoniae* infections, and despite limitations of specificity, the CFT, ELISA, or hemolysin neutralization assays have been useful for the serodiagnosis of *A. pleuropneumoniae* on a herd basis. However, serological results are often nonspecific due to cross-reactions between different serovars of *Actinobacillus* spp., reactions with nonpathogenic *Actinobacillus* species, or other nonrelated bacteria (1, 7, 16, 36). Laboratory tests must be both sensitive and specific. False-positive cases must be avoided to prevent unnecessary disruption in production flow, while false-negative results are problematic because *A. pleuropneumoniae* may be introduced into a naïve population. Despite recent efforts to get a reliable *A. pleuropneumoniae* serological diagnostic test as a basis for success in control and eradication programs, there is still not a single serological test or a perfect combination of assays to identify and differentiate all the *A. pleuropneumoniae* serovars with good sensitivity (3). Because of the lack of sensitivity and specificity of the current serologic tests for *A. pleuropneumoniae* and the technical difficulties in testing a large number of blood serum samples for running multiple tests, a 4-plex FMIA was developed for the simultaneous detection of IgG antibodies against toxins ApxI, ApxII, ApxIII, and ApxIV in a single step. This assay was subsequently used to characterize the serologic anti-Apx toxin antibody profiles in samples with known and unknown *A. pleuropneumoniae* exposure status (3).

Although microsphere-based flow cytometric arrays have been extensively promoted to have broad applications (26, 37), this technology has only been gradually introduced in recent years as a new diagnostic/detection tool for pig diseases, such as *Erysipelothrix rhusiopathiae* (32), porcine reproductive and respiratory syndrome virus, and porcine circovirus (38, 39). To our knowledge, the present study is the first of its kind to describe the use of a 4-plex FMIA for the simultaneous detection of circulating antibody bodies to the *A. pleuropneumoniae* Apx toxins in swine populations.

The potential applications of the novel 4-plex FMIA for anti-Apx-IgG was particularly evident in the experimental samples based on the high sensitivity and capacity for species-specific *A. pleuropneumoniae* serodetection compared to any combination of ELISAs and CFTs used as a reference. The 4-plex FMIA was able to detect, in one assay and in a single sample, specific seroconversion against all *A. pleuropneumoniae* serovars included in the present study, including in seven animals where serocconversion to *A. pleuropneumoniae* was not detected by any of the reference tests used previously. Similar to previous reports (12), seroconversion against ApxI was detected by FMIA in *A. pleuropneumoniae* serovars 1, 5 (only on dpi 7), 9, 10, and 11. However, contrary to expectations, anti-ApxI IgG was not detected in the only pig inoculated with serovar 14 that was included in the study. This animal, which was excluded in a previous study (3), showed a clear and unexpected seroconversion against ApxI and ApxII, and a low and late (by 21 to 28 dpi) seroconversion against ApxIV, perhaps indicating cross-contamination between the testing rooms. In addition, anti-ApxII IgG antibodies were detected in serovars 1 to 4, 6 to 12, and 15, as expected (12), but not in serovar 5, while anti-ApxIII IgG antibodies were detected in serovars 2, 3, 4, 6, 8, and 15, as expected (12). It has been reported that *A. pleuropneumoniae* field strains produce either ApxI, ApxII, ApxII, or ApxIII (40). In line with other publications (12, 41), nonspecific seroconversion against ApxI was detected in serovars 3, 4, 8, and 12, against ApxII in serovars 10 and 14, and against ApxIII in serovars 1, 7, 9, 10 to 12, and 14. In this regard, it has been reported (9) that the ApxI, ApxII, and ApxIII toxins are very closely related, where ApxIII is more related to ApxI (50%) than to ApxII (41%), and ApxI and ApxII are the least related members (37%) of this toxin family. However, ApxIV is organized differently and is genetically very distant from any of the other known RTX toxins (8). In addition, the ApxI, ApxII, and ApxIII toxins of *A. pleuropneumoniae* were found to be antigenically related, and a serologic correlation between serovar and secretion of exotoxin by ELISA was not established (41). This potential cross-reactivity be-
between the ApxI, ApxII, and ApxIII toxins may explain some of the results obtained in the present study. Alternatively, the pigs might have been infected with a different pathogen that induced Apx-like toxins. All reference strains utilized in this study have been well characterized (40, 42, 43), and therefore, additional confirmation of the expression of the respective toxins was not conducted. However, as the production of apx toxin genes may vary between isolates, it is also possible that this might have contributed to the atypical toxin expression patterns and subsequent anti-toxin antibody detection observed with some of the isolates. The presence of an apx gene does also not always correlate with the expression of the corresponding toxin.

According to the established cutoff, one pig inoculated with serovar 5 and one pig inoculated with serovar 7 showed positive levels of anti-ApxIII IgG antibodies before inoculation, which remained positive and relatively stable after inoculation. In contrast, one pig inoculated with serovar 1 had significant levels of anti-ApxII before inoculation but showed a clear response at 7 dpi. Even though the ApxI, ApxII, and ApxIII toxins have been proven to be of value in diagnostic evaluations and virulence studies (21, 41), cross-reactions with the RTX toxins of other bacteria can be expected (16). In fact, in the present study, seroconversion against the toxins ApxII and ApxIII but not against ApxI and ApxIV were detected in pigs that were experimentally infected with A. suis. Therefore, a serologic test based only on ApxI, ApxII, and ApxIII or different combinations of these will also detect other Actinobacillus spp. different from A. pleuropneumoniae organisms. That issue has been overcome in this novel FMIA approach with the inclusion of the ApxIV toxin, which is produced by all 15 serovars of A. pleuropneumoniae and is specific for infections with that species (20).

Contrary to expectations, a specific but low seroconversion rate against ApxIV was detected with serovars 1, 3, and 7 in the vaccinated pigs. The expression of the apxIV has long been considered to be strictly induced in vivo, so pigs immunized with inactivated vaccines would not generate antibodies against ApxIV (11). Therefore, ApxIV antigen has been used to differentiate infected from vaccinated animals (DIVA), as it is immunogenic, specific to A. pleuropneumoniae, and encoded by all serovars (44). However, recently, an ApxIVA protein was identified for the first time from an in vitro growth of A. pleuropneumoniae as part of a subunit vaccine (45). Furthermore, in line with a previous study using ELISA and CFTs (3), a poor or absent seroconversion to ApxII and ApxIII, or ApxI, respectively, was reported for vaccinated pigs by FMIA. Slight seroconversion against ApxII was detected in pigs vaccinated with serovar 5 and against both ApxII and ApxIII in pigs vaccinated with serovar 7. In a previous study, convalescent-phase serum samples of pigs infected with serovar 7, which is thought to exclusively express the apxII gene, allowed detection of toxins ApxI and ApxIII of a subunit vaccine in which ApxII was not present (45). These additional observations further confirm the cross-reactivity between the A. pleuropneumoniae Apx toxins. Due to the great sensitivity displayed by the 4-plex Apx FMIA for specific A. pleuropneumoniae detection, we speculate that the low number of positive samples in the vaccinated pigs is likely due to vaccine composition or preparation.

In field samples, an increase in A. pleuropneumoniae seroprevalence with increasing age was observed through an assessment of anti-ApxIV-specific antibodies by FMIA (Fig. 1A). As expected, FMIA was much more sensitive than ELISA-3 (which also targets ApxIV antibodies) and had an overall moderate agreement, which became poor in older pigs despite the assumption that the prevalence of A. pleuropneumoniae is higher in older pigs. Interestingly, FMIA and ELISA-3 had better agreement with the field samples than with the experimentally derived samples. However, the antibody pattern was different for ApxI, ApxII, and ApxIII, where the seroprevalence decreased gradually with age, reaching the lowest antibody level in grow-finish pigs, but then increased thereafter, reaching peak levels in the adult pigs. Because antibodies against ApxI, ApxII, and ApxIII may also be the result of infection with other bacterial species, and specific information on the A. pleuropneumoniae statuses of the farms where field samples were obtained were not available, the positive results for ApxI, ApxII, and ApxIII in the first stages of production are likely due to passively derived antibodies and/or cross-reactivity with RTX toxins from other bacterial species. The swine nasal cavities and tonsils are heavily colonized with several members of the commensal flora, such as Actinobacillus minor, Actinobacillus porcinus, and Actino- bacillus indolicus (46), as well as with other nonpathogenic Actino- bacillus isolates antigenically and biochemically similar to A. pleuropneumoniae and with an atypical toxin profile (1). For instance, Actinobacillus rossii produces variants of the ApxIII toxin, either alone or in combination with ApxII (7, 47), while A. suis produces RTX toxins that are identical to ApxII and ApxI (40, 48). In addition, the Apx toxins are related to RTX toxins from other species, such as Hly of E. coli and the leukotoxins of Pasteurella haemolytica and Actinobacillus actinomyctematum (9). Moreover, since the Apx antigens were produced using E. coli as the expression system, which also produces a very-well-characterized RTX toxin, this may be an additional source of cross-contamination, making these Apx toxins suitable candidates for use in a eukaryotic rather than a bacterial expression system. RTX toxins are also predominantly found in Pasteurellaceae associated with pathogenic representatives (8), whereas the Pax toxin is present in Pasteurella mairii and Pasteurella aerogenes, showing a high DNA and protein sequence similarity as well as immunological cross-reactivity with ApxIII (49). Although most of these bacterial species probably are not pathogenic in pigs, they may interfere with A. pleuropneumoniae diagnosis.

In the present study, anti-ApxIV antibodies were never detected alone but rather together with antibodies against any combination of the other three Apx toxins. In addition, the serological profiles for ApxI, ApxII, and ApxIII are indicative of the toxicity and virulence of A. pleuropneumoniae strains (5). Therefore, the simultaneous detection of antibodies against the four Apx toxins in one test may be extremely valuable for confirmation of A. pleuropneumoniae infection and to obtain the Apx toxin expression profile for future epidemiology and virulence studies. However, due to the potential cross-reactivity between ApxI, ApxII, and ApxIII toxins with other bacterial RTX toxins in field samples with unknown exposure status, the inclusion of additional targets to the current 4-plex Apx FMIA, such as serovar-specific long-chain lipopolysaccharides, as well as further studies on recombinant ApxIV variants, may be helpful. The novel approach described herein therefore represents the first step in the development of a wider multiplex diagnostic platform for Actinobacillus spp. in pigs. Future expansion of the current test might include the screening of different overlapping oligopeptides within the Apx toxins, as well as serovar-specific antigens and/or antibodies de-
rived from capsular polysaccharides and long-chain lipopolysaccharides. Overall, the results of this study demonstrate that the developed novel 4-plex ApxF MIA is an efficient and reliable test for the serologic detection of *A. pleuropneumoniae* infection, with an overall sensitivity that is greater than those of the tests currently available in most diagnostic labs.

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