

Lipopolysaccharide-Based Enzyme-Linked Immunosorbent Assay for Experimental Use in Detection of Antibodies to *Lawsonia intracellularis* in Pigs

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An enzyme-linked immunosorbent assay (ELISA) for *Lawsonia intracellularis* was developed and compared with a whole-cell antigen-based immunofluorescence antibody test (IFAT). The antigen-containing lipopolysaccharide (LPS) was derived from Percoll gradient purified cultures of *L. intracellularis* by using a modification of the Westphal hot phenol procedure. The antigen was bound directly to polystyrene 96-well microtiter plates, and the assay was performed in an indirect ELISA format. Specificity and sensitivity values based on 80 known positive and 80 known negative serum samples from controlled experimental trials were 93.7% and 88.7%, respectively. Serological results from a controlled *L. intracellularis* challenge exposure study confirmed the high specificity and sensitivity of this assay (100% and 99.5%, respectively). Comparisons between the LPS ELISA and the IFAT in detecting anti-*Lawsonia* antibodies in this controlled study revealed significantly more LPS ELISA-positive pigs than IFAT-positive pigs on days 21, 28, 35, and 42 ($P = 0.003, 0.030, 0.002, \text{ and } 0.006$, respectively). This indirect ELISA (LPS ELISA) test is an improved method of detecting antibodies in pigs soon after exposure to *L. intracellularis*, regardless of isolate type (vaccine or wild type) in experimental studies. The LPS ELISA may be used as a tool to support future research trials on vaccine efficacy and to further understand the immune response induced by *L. intracellularis*.

Proliferative enteropathy (PE) is a common enteric disease of pigs after weaning that is caused by the obligate intracellular bacterium, *Lawsonia intracellularis* (20, 21). The infection and disease are widespread in pig farms across America and Europe, and prevalence among groups of pigs on these affected farms can be over 30% (4, 23, 29, 31). This leads to a considerable economic impact of the disease, due to diarrhea, weight loss, and subclinical illness in growing pigs (22, 31). Since the identification of *L. intracellularis* as the cause of PE in 1993, a number of studies aimed at establishing the best diagnostic methods for identifying *Lawsonia* exposure in live animals have been conducted. These have focused on DNA detection via PCR of feces and whole-cell immunoassays (8, 12, 13, 15, 17), due to the extreme difficulty of isolation of the obligate intracellular *L. intracellularis* from the contaminated environment of feces (13, 17, 18). In situations where samples of ileum are available, immunohistochemistry (IHC) is considered to provide the criterion-referenced measure or “gold standard” for assessment of the actual infection status of an individual pig (9, 16, 19, 26, 28).

PCR testing of fresh feces involves considerable laboratory effort and cost to extract amplifiable bacterial DNA from each sample (9, 11, 13, 15). False positives due to pre-laboratory sample contamination during the collection of numerous samples from a group of pigs or due to contamination during the laboratory testing phase may occur. False negatives due to the

regular presence of PCR inhibitors in feces may also occur (9, 10, 11). Serologic testing methods have therefore also been widely explored for detecting *L. intracellularis* exposure of pigs. Indirect immunofluorescence or immunoperoxidase assays have been used to examine antibody responses of pigs infected experimentally with *L. intracellularis* in virulent challenge exposure studies and of pigs with PE from farms (3, 4, 7, 11, 14, 29). An indirect enzyme-linked immunosorbent assay (ELISA) was developed previously for testing pig serum antibodies, with crude antigen derived directly from pig intestines affected with PE (12). However, the antigen used in that study was not fully characterized for *L. intracellularis* content. The development of a specific *L. intracellularis* antigen-based ELISA would therefore be of considerable benefit in improving the feasibility of a more universally available and standardized diagnostic assay to study the epidemiology of this economically significant disease.

We describe the development of an ELISA for detecting *L. intracellularis* infection based on a lipopolysaccharide antigen extract in an indirect ELISA format.

MATERIALS AND METHODS

Bacterial antigen preparation. The lipopolysaccharide (LPS) used in this study was derived from *L. intracellularis* isolate 15540. This isolate was acquired from a Danish sow affected with acute hemorrhagic proliferative enteropathy (confirmed by routine histology and immunohistochemistry staining techniques) whose intestines were cocultured to obtain a pure culture of *L. intracellularis* by methods previously described (18, 21). Multiple 30-liter batches of *L. intracellularis* 15540 (ATCC PTA-4927) were propagated using fresh McCoy cell (ATCC 1696) suspensions in bioreactors (Applicon, Inc., Foster City, CA). Active cultures were allowed to reach 80 to 100% cell infectivity and then were harvested by centrifugation using an Avanti Beckman J-201 centrifuge with JA-10 rotor at $17,000 \times g$ for 15 min at 4°C. The supernatants of each batch were discarded, and cell pellets containing both harvested extracellular *L. intracellularis* and McCoy

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cells infected with *L. intracellularis* were resuspended in 30 ml of sterile 0.2 M phosphate-buffered saline (PBS) at pH 7.3 and stored at -80°C .

For purifying *L. intracellularis* from McCoy cells, a discontinuous Percoll gradient was prepared following methods previously described, with slight modifications (12). Briefly, 225 ml of Percoll (Amersham Biosciences, Uppsala, Sweden) was mixed with 260 ml of distilled water and 15 ml of 5 M NaCl to form the stock Percoll gradient. Harvested *L. intracellularis* culture was passed at least 20 times through a 25-gauge needle, and 5 ml of this bacterial-McCoy cell homogenate was mixed with 25 ml of the stock Percoll gradient in polycarbonate tubes. The tubes were then centrifuged at $37,000 \times g$ for 1 h at 4°C . The ensuing suspension contained scattered cellular debris in the upper 50% of the tube, while one distinct cellular banding pattern was visualized at a buoyant density of 1.075 g/ml. This band was carefully collected into a 5-ml polypropylene pipette and transferred to new tubes containing 20 ml of sterile PBS. These tubes were centrifuged (Avanti Beckman J-20I, JA-17 rotor) at $37,000 \times g$ for 15 min at 4°C , and this washing process was repeated three times to remove Percoll material from each sample. After the final centrifugation step, the bacterial pellets were suspended in sterile PBS, aliquoted into 1.8-ml Cryovial tubes, and stored at -80°C . Dark field microscopy of sampled material confirmed highly concentrated tiny curved rods and the absence of intact McCoy cells.

The LPS component was extracted from this purified *L. intracellularis* 15540 antigen using a hot aqueous phenol method (Westphal method) with slight modifications (34). Briefly, 4.5 ml of a preheated purified *L. intracellularis* suspension was gently mixed in a tube containing 4.5 ml of a 90% (vol/vol) hot aqueous phenol suspension for 25 min at 65°C and then cooled overnight at 4°C . Each tube was centrifuged at $7,700 \times g$ for 25 min at 4°C , and the LPS-containing supernatant fluid contained in the upper portion of the biphasic aqueous layer within each tube was retained and transferred into presterilized dialysis tubing. The tubing was then dialyzed repeatedly against cold reagent-grade (reverse osmosis) water for 48 h to remove the phenol. The *L. intracellularis* LPS extract was collected and centrifuged at $10,000 \times g$ for 15 min at 4°C to remove any remaining particulates and stored in sterile vials at -80°C .

Antigen characterization. The LPS extract was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 4 to 12% Bis-Tris gel in MOPS (morpholinepropanesulfonic acid) running buffer (Invitrogen) and compared to Percoll-purified whole-cell *L. intracellularis* and uninfected whole McCoy cells. Samples for SDS-PAGE were prepared by diluting each 1:2 into $4 \times$ lithium salt dodecyl sulfate denaturing buffer (Invitrogen) and incubated in an 85°C water bath for 10 min. Gels were periodate silver stained (30) using a slight modification of the commercially available Bio-Rad silver stain. Briefly, gels were oxidized with 0.7% periodic acid immediately after fixing and prior to the second fixation step (10% ethanol and 5% acetic acid), and an additional reduction step with 160 μM dithiothreitol was included before the silver reagent step. In other experiments, the LPS extract was subsequently transferred from unstained SDS-PAGE gels to polyvinylidene difluoride (Invitrogen) membranes for Western blot analysis. The separated molecules were electrophoretically transferred in Towbin buffer using a Novex blot module at a constant voltage of 30 V for 1 h. After transfer, the blots were cut into strips and blocked for at least 1 h in 50 ml of a blocking solution consisting of Tris-buffered saline with 0.05% (vol/vol) Tween 20 (TTBS) and 2% (wt/vol) nonfat dry milk (Bio-Rad). Blots were incubated with numerous primary antibody sources and dilutions, as follows: (i) LPS ELISA-positive control hyperimmunized pig serum (1:2500), (ii) LPS ELISA-negative control pig serum (1:2500), (iii) swine anti-*Lawsonia* convalescent-phase serum (1:10, 1:20, 1:100), and (iv) swine *Lawsonia*-negative convalescent-phase serum (1:100) to illustrate the specificity of anti-*Lawsonia* antibodies to the primary antigen of the extract, *Lawsonia* LPS. The primary antibody dilutions used for this Western analysis were similar to those used for the LPS ELISA. The primary antibodies were diluted in the blocking reagent described above and incubated with each respective membrane at room temperature for 1 h. The blots were washed three times for 2 min at room temperature in TTBS, after which the membranes were incubated in 50 ml of the secondary antibody conjugate (goat anti-swine horseradish peroxidase [HRP]; Kirkegaard and Perry Laboratories, Inc.) at a 1:1,000 dilution in blocking reagent for 1 h at room temperature. The blots were washed twice in TTBS and once in PBS prior to incubation at room temperature for 30 min in 10 ml of Opti-4CN substrate (Bio-Rad) solution. The colorimetric reaction was stopped by rinsing the blots with reagent-grade water.

Lastly, the LPS extract was analyzed for a specific concentration by a bacterial endotoxin test with a *Limulus* amoebocyte lysate assay (Endosafe). The concentration of LPS within the extract was expressed as endotoxin units (EU) per ml using the kinetic chromogenic method as described in the manufacturer's instructions.

Optimization of the LPS ELISA. The ELISA was optimized following standard assay development procedures described elsewhere (6, 27). Concentrations of key reagents used in this LPS ELISA, such as the primary antigen (LPS extract), primary antibodies, and HRP-conjugated secondary antibodies, were optimized to achieve a desired and consistent working dilution using checkerboard titrations (6, 27). Other influencing factors key to assay success, such as blocking and wash buffers, substrates, incubation temperatures, and duration of incubation time periods, were also evaluated according to standard procedures to achieve optimum levels (6, 27).

LPS ELISA for anti-*Lawsonia* IgG antibodies. The *Lawsonia intracellularis* LPS, at a concentration of 34.8 EU/ml, was used to coat Immulon 2HB plates (Dynex) at 100 μl /well at a 1:1,000 dilution in 0.05 M sodium carbonate coating buffer, pH 9.6 (2), and incubated at 20°C for 24 h. Each plate was then washed in a buffer containing 0.05% Tween 20 (vol/vol), 0.137 M NaCl, 0.005 M KCl, 0.009 M Na_2HPO_4 , and 0.001 M KH_2PO_4 (pH 7.2 to 7.4) in distilled water. Antigen-coated plates were then blocked at 300 μl /well with a blocking buffer containing 5% (wt/vol) nonfat dry milk (Bio-Rad) in SeaBlock (Pierce Biotech) at 4°C for 24 h to prevent the nonspecific binding of test serum to the plates. Each plate was then washed for three cycles as described above. Each test and control serum sample was prediluted 1:40 in blocking buffer. Fifty μl per well of each diluted serum sample was incubated with antigen at 37°C for 1 h. After three washing steps, 50 μl /well of a 1:500 dilution of goat anti-swine immunoglobulin G (IgG) heavy and light chain-specific HRP conjugate (Kirkegaard and Perry Laboratories, Inc.) in blocking buffer was added and incubated at 37°C for 1 h. After three washing steps, 50 μl /well of peroxidase color substrate (3,3',5,5'-tetramethylbenzidine) was applied to each plate and incubated at 20°C for 5 min and then stopped with a 2 M H_2SO_4 solution. Absorbance values at a wavelength of 450 nm (A_{450}) were obtained for each sample well. A range of control serum samples from high- to low-positive and negative antibodies against the *L. intracellularis* LPS extract were generated and included for each plate. Control sera were initially diluted 1:2,560 in blocking buffer and were then serially diluted twofold a total of five times (1:2,560, 1:5,120, 1:10,240, 1:20,480 and 1:40,960) immediately prior to sample testing. Aliquots (50- μl) of each diluted positive and negative control samples were transferred and typically placed in wells A through E in columns 11 and 12 of the test plate. A test plate was considered valid if the coefficient of determination (r^2 value) of these standards' A_{450} values was ≥ 0.9 in a linear regression analysis (Microsoft Excel), with empty wells reading blank.

Serum samples derived from clinical pig trials. Serum samples were obtained from pigs in the following studies for use in validating the LPS ELISA.

Study 1. Two 3-week-old *Lawsonia*-negative pigs were hyperimmunized for antibodies to the *L. intracellularis* LPS extract by deep intramuscular injection with 2 ml of the *L. intracellularis* LPS extract mixed 1:2 with a commercial Freund's incomplete adjuvant (Sigma Aldrich). Another control 3-week-old pig received a placebo injection of cell culture medium containing 5% (vol/vol) bovine serum, mixed 1:2 with this adjuvant. Booster injections of each inoculum were administered 3 and 6 weeks post-initial inoculation (p.i.), and final specimen collections were at 8 weeks p.i. Serum samples from each pig were collected on days 0, 21, 42, and 59 of the study and tested for the presence and concentration of anti-*Lawsonia* IgG antibodies by using an indirect immunofluorescence assay incorporating *L. intracellularis* whole-cell antigen (immunofluorescence antibody test [IFAT]) as described previously (15).

Study 2. Challenge exposure studies with *L. intracellularis* isolates were conducted with *Lawsonia*-negative 6- to 9-week-old pigs as described previously (16, 33). Serum samples from the virulent *L. intracellularis* challenge-exposed and strict control pigs (nonvaccinated, nonchallenged pigs) in these studies were tested to investigate the cutoff limits for anti-*Lawsonia* LPS antibody positive/negative absorbance values. The *Lawsonia* IFAT was also conducted with these samples.

Study 3. A "proof of concept" vaccine efficacy study was conducted following good clinical practice guidelines as previously reported (16). This study utilized conventional pigs that were *Lawsonia*-negative at the time of vaccination. On day 0 of the study, vaccinates ($n = 15$) were given an oral 2-ml dose of Enterisol Ileitis (Boehringer Ingelheim Vetmedica, Inc.) per the manufacturer's label instructions, while pigs in the challenge control group ($n = 10$) were given an equivalent dose of placebo consisting of uninfected McCoy cells in growth media. Strict control pigs were not given vaccine, placebo, or virulent challenge during the study and were kept in separate pens to ensure biosecurity among treatment groups. On day 21, vaccinated and challenge control pigs were given a 10-ml intragastric dose of virulent *L. intracellularis* isolate N10194 at $10^{7.7}$ tissue culture infective dose 50% end point (TCID₅₀) per ml. On day 42 of the study, pigs were humanely euthanized, necropsied, and evaluated for *Lawsonia*-specific gross and microscopic lesions. Average gross and microscopic lesion scores (immunohistochemistry), average daily weight gains, seroconversion rates (IFAT), *L. intra-*

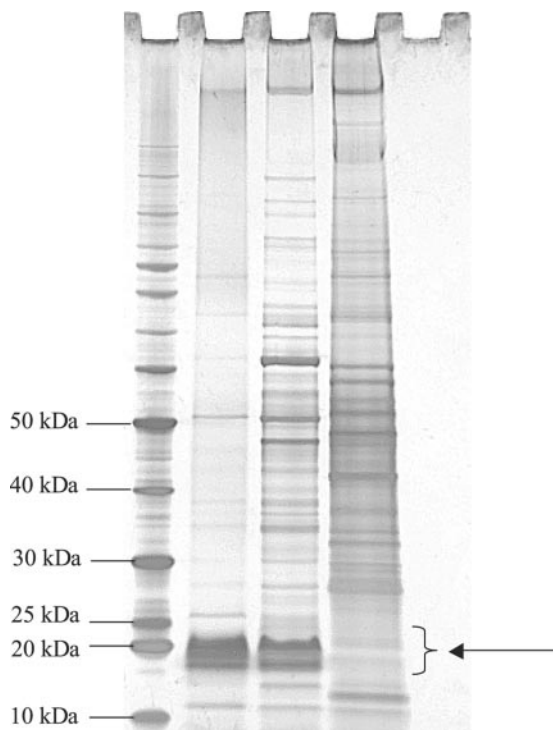


FIG. 1. SDS-PAGE-periodate silver staining of *L. intracellularis* LPS. First lane, 10- to 220-kDa benchmark prestained protein marker (Invitrogen); second lane, *L. intracellularis* LPS extract; third lane, whole-cell *L. intracellularis* 15540; fourth lane, uninfected McCoy cells; fifth lane, blank. The arrow indicates the migration range of *L. intracellularis* LPS.

cellularis fecal shedding rates (PCR), and average daily clinical scores after challenge of this clinical trial were previously reported (16). Serum samples were subsequently tested with the LPS ELISA for direct comparison to the IFAT for differences in detecting anti-*Lawsonia* antibodies in vaccine- and challenge-exposed pigs.

Statistical analysis. Statistical comparisons in seroconversion among assays and treatment groups utilized Fisher's exact *t* test and a two-sample test of proportions (5, 25). Statistical determinations of positive and negative A_{450} cutoffs, as well as assay sensitivities, specificities, and 95% confidence intervals and the areas under the receiver operating characteristic (ROC) curve were conducted with ROC curve analysis using Medcalc version 7.2.1.0. These additional parameters were observed to identify the assay's ability to detect *Lawsonia*-specific antibodies in pigs and to gauge the assay's ability to distinguish between *Lawsonia*-exposed and *Lawsonia*-naïve pigs.

RESULTS

Characterization of the *L. intracellularis* LPS extract as the primary ELISA antigen. The visualization of the *L. intracellularis* LPS component by the SDS-PAGE-periodate silver staining of the LPS extract revealed a glycosylated molecule of approximately 18 to 25 kDa (Fig. 1). Glycosylation was confirmed by the reddish staining imparted on the species via the reaction of aldehyde groups formed by periodate oxidation of the sugars with the silver reagent. This LPS fraction was differentially stained reddish brown to specifically identify glycosylated moieties in comparison to other extracted components. The LPS was observed with the samples containing the LPS extract and whole cell *L. intracellularis* and not with the uninfected McCoy cell sample, suggesting that this molecule is of

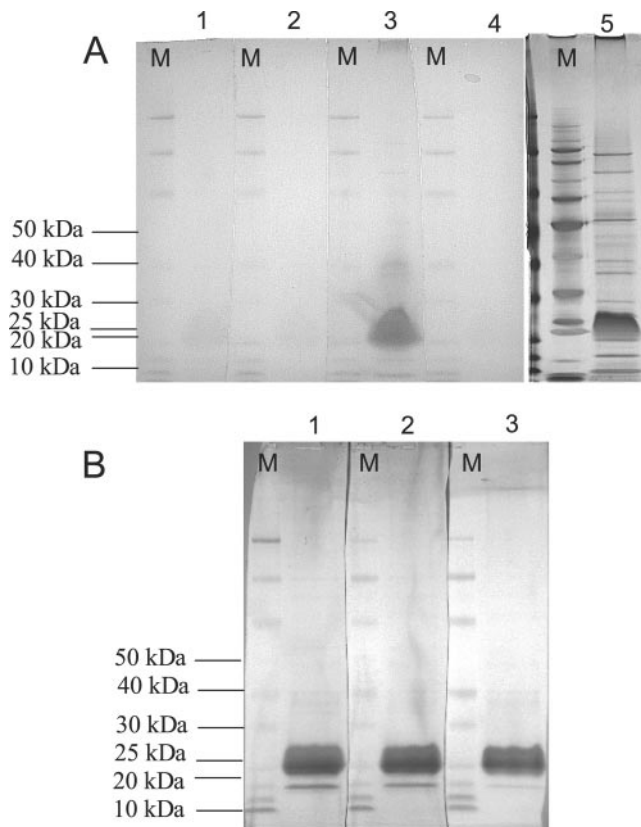


FIG. 2. Western blots of *L. intracellularis* LPS using ELISA control or positive and negative convalescent swine sera. Each gel lane was loaded with the same amount of LPS material, and replicate lanes were cut into strips after transfer to polyvinylidene difluoride for incubation with different primary antibodies. Secondary antibody in each case was goat anti-swine-HRP (1:1,000). M, molecular weight marker. (A) Lane 1, strict control negative serum (1:100); lane 2, ELISA negative control serum (1:2,500); lane 3, ELISA positive control serum (1:2,500); lane 4, goat anti-swine-HRP conjugate only (1:1,000); lane 5, silver-stained SDS-PAGE gel. (B) Primary antibody was swine anti-*Lawsonia* convalescent-phase serum at different dilutions. Lane 1, 1:10; lane 2, 1:20; lane 3, 1:100.

prokaryotic origin. The periodate silver-stained gel also revealed the presence of other, higher-molecular-weight proteins that seem to appear at lower concentrations compared to the LPS extract. These extraneous proteins perhaps are due to inadvertent carry-over of particulates from the biphasic layer during the purification step, or they could be the results of incomplete degradation of proteins during the hot aqueous phenol phase of the LPS extraction.

Western blots of the LPS extract were performed to determine the specificity of anti-*Lawsonia* antibodies to the LPS component and to assess the degree of cross-reactivity to the extraneous proteins (Fig. 2). Results of the Western blots demonstrated that the LPS ELISA negative control (1:2,500) and *Lawsonia*-negative pig sera (1:100) did not react to any of the extraneous proteins in the LPS extract and reacted very lightly (weak signal strength) to the LPS component itself. Positive LPS ELISA control sera (1:2,500) reacted very strongly (strong signal strength) to the LPS component and, to a lesser extent, the extraneous proteins. Slight cross-reaction of the positive

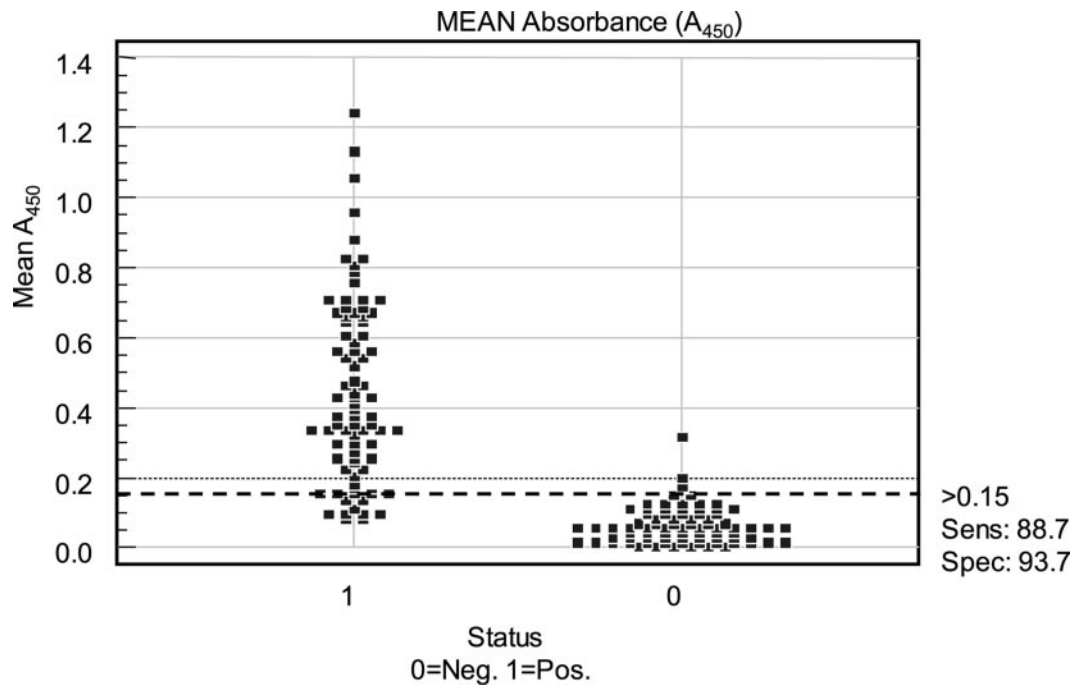


FIG. 3. Scatter plot of the mean A_{450} values for anti-*Lawsonia* antibody-positive and -negative serum samples. The y axis depicts mean A_{450} values, and the x axis depicts sample set identification. 0, negative (Neg.); 1, positive (Pos.) for anti-*Lawsonia* antibodies. The dashed black line at the A_{450} of 0.15 signifies the positive/negative cutoff value. Sens, sensitivity; Spec, specificity.

control was expected, since hyperimmunized pigs would have generated antibodies to both the LPS component and the extraneous proteins. However, the Western signal for this particular interaction was very light, making up <1% of the total signal strength compared to the target LPS antigen. Additionally, Western analysis of the LPS extract to *Lawsonia*-positive convalescent pig serum at dilutions of 1:10, 1:20, and 1:100 revealed strong reactions to the LPS component, whereas very light reactions were noted with the higher-molecular-weight extraneous proteins at 1:10 and 1:20 dilutions only (<1% of total signal strength). An extraneous protein of lower molecular weight than the LPS component (approximately 15 kDa) did react to *Lawsonia*-positive convalescent pig sera but represented only ~9% of the total signal strength of the reaction compared to the LPS component.

Results from the bacterial endotoxin test for quantifying the amount of endotoxin in the purified sample of *L. intracellularis* LPS extract revealed an endotoxin level of 34,750 EU/ml. Furthermore, the reagent-grade water used to store the LPS extract contained <0.05 EU/ml, thus indicating that the buffer was endotoxin free and provided no interference in obtaining the true endotoxin concentration of the LPS extract.

Individual LPS ELISA plate validation via hyperimmunized pig sera. The positive LPS ELISA control serum was generated from two 3-week-old *Lawsonia*-negative pigs hyperimmunized with *Lawsonia* LPS extract. The negative control serum was generated from one 3-week-old pig receiving adjuvant alone. Pigs that received an LPS extract inoculation were IFAT positive on days 21, 42, and 59 of the study, with mean anti-*Lawsonia* antibody titers of 1:200, 1:16,000, and 1:16,000, respectively. Reactivity of the positive and negative control sera

against the *Lawsonia* LPS antigen extract (1:1,000) in the LPS ELISA plates was confirmed by performing twofold titrations in blocking buffer of each control across the plate. The positive control A_{450} value at a 1:40 dilution (optimal detection antibody dilution for this assay) was 1.9 and was determined to have a total anti-*Lawsonia* LPS endpoint titer of 1:10,000 to 1:20,000 by the LPS ELISA. The negative control A_{450} value at a 1:40 dilution was <0.2. Linear regression analysis of the positive control demonstrated an r^2 value of 0.96. Based on the linearity of the positive control, an individual LPS ELISA assay is considered valid in detecting *Lawsonia*-specific antibodies when each plate demonstrates an r^2 value of 0.9 or greater.

Determination of cutoff limits. One hundred sixty pig serum samples from two *L. intracellularis* challenge exposure studies were evaluated to determine the anti-*Lawsonia*-positive and -negative antibody cutoff limits for the LPS ELISA assay. In addition, these serum samples were used to determine assay specificity, sensitivity, 95% confidence interval, and the area under the ROC curve analysis. Eighty pigs were previously identified to be positive for *L. intracellularis* by both IFAT and tissue immunohistochemistry analysis (16, 33). Another 80 pigs from strict control (nonvaccinated, non-challenge-exposed) and negative control (received a non-*Lawsonia* placebo) groups were previously identified to be negative for *L. intracellularis* by the same detection methods (16, 33).

A ROC curve analysis, a graph that plots the true positive rate in function with the false-positive rate at different cutoff points, identified the critical cutoff criterion for these samples to be an A_{450} of ≥ 0.15 (Fig. 3). This value was rounded to the nearest tenth of an A_{450} value, to 0.2, to simplify future interpretations of LPS ELISA results. Therefore, pig serum with an

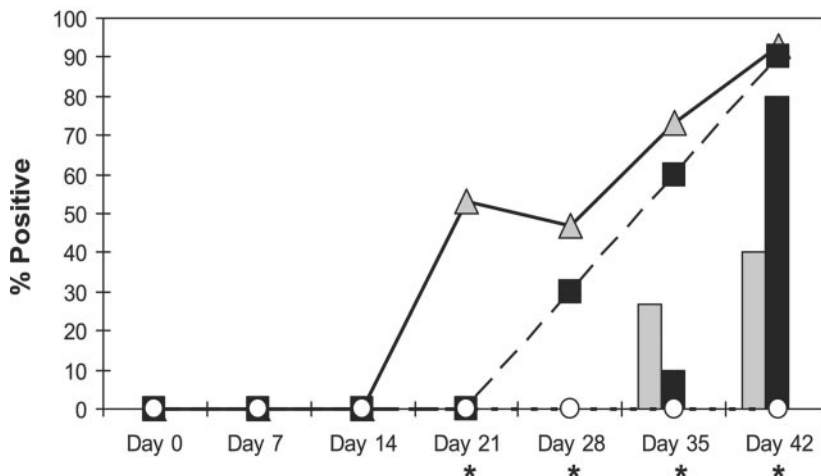


FIG. 4. IFAT versus indirect LPS ELISA; rates of seroconversion to *L. intracellularis* among treatment groups. *, significant differences in seroconversion among groups and assay types (chi-square/Fisher's exact test and two-sample test of proportions, $P < 0.05$). The bar graph represents the mean percentage of IFAT positives for treatment groups throughout the study. Vaccinates, grey bars; challenge controls, black bars; strict controls, white bars (not visible, 0%). The line graph represents the mean percentage of LPS ELISA positives for treatment groups throughout the study. Vaccinates, solid line with grey triangles; challenge controls, dashed line with black squares; strict controls, dashed line with white circles; x axis, each day of the study that serum samples were tested for anti-*Lawsonia* IgG; y axis, mean percentage of anti-*Lawsonia* antibody positive pigs.

A_{450} value of 0.2 or lower is considered *Lawsonia* antibody negative, whereas serum with an A_{450} value of greater than 0.2 is considered *Lawsonia* antibody positive.

In addition, the ROC analysis revealed that the specificity of this assay to detect anti-*Lawsonia* antibodies in pigs was 93.7%, and the sensitivity was determined to be 88.7% (Fig. 3). The 95% confidence interval for this assay was 0.933 to 0.991, and the ROC under the curve analysis revealed a value of 0.972, with a standard error of 0.013.

LPS ELISA validation in a controlled, *L. intracellularis* challenge-exposed study. Pigs in the strict control group remained *L. intracellularis* IFAT and LPS ELISA negative through the study. From day 0 (vaccination) to day 14 (1 week before challenge) of the study, vaccinated and unvaccinated challenge control groups were IFAT and LPS ELISA negative for anti-*L. intracellularis* antibodies. On day 21 (challenge inoculation) of the study, all groups were IFAT negative (0%), whereas 53% of the vaccinated pigs exhibited LPS ELISA positive results. One week after challenge (day 28), again only the LPS ELISA was able to detect specific seroconversion to anti-*Lawsonia* antibodies in both the vaccinated and challenge control groups (47% and 30%, respectively). On days 35 and 42 of the study, the number of pigs in both the vaccinated and challenge control groups gradually increased in seroconversion due to virulent *L. intracellularis* exposure. Serological results from days 35 and 42 in the vaccinated pigs were 26.7% and 40% IFAT positive and 73% and 93% LPS ELISA positive, respectively. The challenge control group was 10% and 80% IFAT positive and 60% and 90% LPS ELISA positive on days 35 and 42, respectively.

The seroconversion rates based on the LPS ELISA identified significant ($P < 0.05$) increases in the percentage of anti-*Lawsonia* antibody positives in the vaccinate group compared to challenge control and strict control pigs at day 21 and compared to strict control pigs only on days 28, 35 and 42 of the

study. Nonvaccinated challenge controls were significantly higher in the percentage of LPS ELISA positives than the strict controls on days 35 ($P = 0.001$) and 42 ($P < 0.0001$) of the study. No significant differences between vaccinates and challenge controls on days 35 and 42 were noted. Significant ($P = 0.02$) increases in the percentage of IFAT positives to *L. intracellularis* challenge exposure were evident for vaccinated pigs compared to strict control pigs on day 35 of the study. Vaccinates and challenge controls were significantly ($P = 0.003$) more IFAT positive than the strict control group on day 42 of the study. No significant differences in the percentage of IFAT positives were evident between vaccinates and challenge controls on days 35 and 42. Group comparisons in seroconversion rates between assays and treatment groups are summarized in Fig. 4.

Comparisons between the LPS ELISA and the IFAT in detecting anti-*Lawsonia* antibodies in this study revealed significantly more LPS ELISA-positive pigs than IFAT-positive pigs on days 21, 28, 35, and 42 ($P = 0.003, 0.030, 0.002,$ and 0.006 , respectively).

The ROC analysis of the results from this study revealed the specificity of the LPS ELISA to be 100% and the sensitivity 99.5%. The 95% confidence interval was 0.976 to 0.999, and the ROC under the curve analysis revealed a value of 0.995, with a standard error of 0.007.

DISCUSSION

We have developed a novel LPS-based indirect ELISA that specifically detects anti-*Lawsonia* IgG in the sera from *Lawsonia*-negative pigs exposed to a modified live vaccine and/or virulent challenge. This immunoassay could improve the early detection of *L. intracellularis* exposure in pigs by providing a user-friendly and time-efficient research tool for use in controlled clinical evaluations of pigs. The incorporation of this

Lawsonia-specific and -sensitive antibody-antigen reaction into direct or sandwich ELISA approaches may allow other novel immunoassays to be developed for research purposes. Modifications to the conjugate in this LPS ELISA would also allow for detection of other anti-*Lawsonia*-specific IgA or IgM in pigs or antibodies in other host species. Modifications to the method for preparation of the test sample may also allow testing of tissue, feces, or sow milk samples for the test antibody.

The use of this type of LPS ELISA format has been successfully deployed in serologic assays for other enteric and intracellular bacteria, such as *Salmonella dublin* and *Brucella* spp. (1, 2, 32). The diagnostic performance of this assay is not expected to be affected by alterations in the antigen's lipid A structure or contaminating outer membrane proteins that may occur in different antigen preparations (1, 2, 24). The standard carbonate buffer coating conditions used in our study are considered suitable for the LPS ELISA format for smooth LPS antigen (2). There was also a good correlation of the LPS ELISA results in a range of field studies for *Salmonella dublin* infection on farms (32), and these results were superior to previous *Salmonella* immunoassay formats (32). The LPS ELISA described here is therefore likely to provide a robust, widely applicable diagnostic screening tool with broad benefits for disease interpretation among groups of pigs.

Current serology-based assays for *L. intracellularis* infection and disease are the IFAT (15) and the immunoperoxidase monolayer assay (7, 8), both incorporating whole-cell antigen on carrier wells in plates or slides. They demonstrate good sensitivity (91% and 89%, respectively) and specificity (100% for both assays) in detecting anti-*Lawsonia* antibodies antemortem in pig serum. However, both assays are probably not sensitive enough to detect anti-*Lawsonia* antibodies in lower concentrations in pig serum during the initial exposure and onset of infection. In addition, these assays rely on highly skilled technicians to accurately conduct the tests and interpret the results. Results are obtained subjectively by spending many hours looking into a microscope, analyzing wells illuminated by UV or natural light, and searching for *L. intracellularis*- or *L. intracellularis*-infected cells stained fluorescent green or enzymatic red, respectively, which represents a "positive" sample. A previous attempt to develop an ELISA for the detection of anti-*Lawsonia* antibodies in pigs failed to produce a sensitive and specific assay, due to its variable and nonspecific quality of antigen (derived directly from pig intestines) and limitations in sampling size related to known disease parameters for test pigs (12).

In the LPS ELISA described here, we used an antigen source consisting of phenol-purified LPS derived from pure cultures of *L. intracellularis*, with a specific cutoff value (A_{450} , 0.200) that has been determined for identifying anti-*Lawsonia* antibody-positive and -negative pig sera. The ability of the LPS ELISA to accurately distinguish *Lawsonia*-exposed pigs has been established by observing the results of the ROC under the curve value and 95% confidence intervals in the controlled challenge exposure study. The strong ROC under the curve value of 0.995 and the 95% confidence intervals of 0.976 to 0.999 identify the LPS ELISA as a highly accurate serological test for detecting anti-*Lawsonia* antibodies in pigs exposed to *L. intracellularis* in these experimental trials. Serological results

from a controlled study of *L. intracellularis*-challenge-exposed pigs reveal the superiority of the LPS ELISA in detecting early antibody onset following vaccine and virulent challenge exposure compared to the IFAT. Significantly ($P < 0.05$) more vaccinated pigs (53%) were LPS ELISA positive prior to receiving a virulent challenge (day 21) than nonvaccinated challenge controls (0%). The LPS ELISA was the only test sensitive enough to detect seroconversion in pigs specifically due to vaccination at 3 weeks p.i. (day 21). The LPS ELISA significantly ($P < 0.05$) detected more anti-*Lawsonia* positives than the IFAT in pigs regardless of treatment groups on the day of challenge (day 21) and 1, 2, and 3 weeks after virulent challenge. The implications for detecting earlier onset of *L. intracellularis* exposure in pigs using the LPS ELISA will allow a more precise and rapid implementation of *L. intracellularis* control strategies, such as vaccination to control and reduce PE.

Percentages of mean LPS ELISA positives tended to be higher for vaccinated pigs (47%, 73%, and 93%, respectively) than for the challenge controls (30%, 60%, and 90%, respectively) each day after challenge exposure, whereas the IFAT results revealed the first evidence of seroconversion in vaccinated pigs at 2 weeks postchallenge (26.7%) and showed a gradual increase to no more than 40% IFAT-positive pigs on the last day of the study. The challenge controls demonstrated sharp increases in the percentages of IFAT positives, with 10% and 80% on days 35 and 42 of the study, respectively. The LPS ELISA seems more sensitive in that it identified more anti-*Lawsonia* positive pigs in the vaccinates than in the nonvaccinates after virulent challenge; this trend differs from those seen in previous studies using the IFAT (16).

Specificity and sensitivity values based on 80 known positive and 80 known negative test samples were 93.7% and 88.7%, respectively. Serum tested from the challenge exposure study confirmed this result by demonstrating similar specificity (100%) and sensitivity (99.5%) in detecting anti-*Lawsonia* antibodies in pigs. Western blot analysis of the LPS extract supports this high specificity in detecting anti-*Lawsonia* antibodies with the LPS ELISA, even though the LPS extract contained extraneous protein carry-over. It appears that at the dilutions used, the reagents of the ELISA are quite specific for what is believed to be a component of *Lawsonia* LPS. The low level of carry-over protein within the LPS extract contains no confounding effects in the assay's ability to specifically detect *Lawsonia*-specific antibodies in pigs. Furthermore, quantification of total protein in the undiluted LPS extract by a BCA protein assay (Pierce Laboratories, Inc.) revealed concentration levels too low (0.11 mg/ml) for establishing adequate antigen-antibody complexes.

Despite the positive results noted in this investigation, there are some factors which limit the application of the described LPS ELISA beyond the research laboratory. Other antigen preparations of differing purities were examined and found not to have sufficient sensitivities and specificities for use in *Lawsonia*-specific research trials (data not shown). The requirement of antigen derived from Percoll gradients (low in or free from eukaryotic cellular debris) and phenol extraction methods make *Lawsonia* LPS antigen availability practical for only limited numbers of assays and probably cost prohibitive for uses beyond research trials. Secondly, limited evaluation of this

assay under field conditions suggests that there may be differences in animal backgrounds and LPS reactivity, especially in adult pigs. If this assay were to be applied as a routine diagnostic assay, it is possible that different cutoff values would be required and sensitivity and specificity would be lower.

The technical advantages outlined above make the LPS-based indirect ELISA test an improved serological method for detection of *L. intracellularis* exposure in pigs compared to the IFAT, because it successfully and accurately identified early-onset exposure to this organism regardless of isolate type (vaccine or wild type) and provided a rapid and user-friendly tool for identifying specific seroconversion to *L. intracellularis* in experimental studies.

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