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Evaluation of a novel enzyme-linked immunosorbent assay for detection of antibodies against *Salmonella*, employing a stable coating of lipopolysaccharide-derived antigens covalently attached to polystyrene microwells

Camilla Wiuff, Eva Stenbæk Jauho, Henrik Stryhn, Lars Ole Andresen, Karina Thaulov, Ulrik Boas, Mogens Havsteen Jakobsen, Peter M. H. Heegaard

Abstract. Polysaccharides derived from *Salmonella typhimurium* lipopolysaccharide (LPS) representing the O-antigen factors 1, 4, 5, and 12 and the O-antigen factors 6 and 7 from *Salmonella choleraesuis* LPS were derivatized with the photoreactive compound anthraquinone and subsequently covalently coupled to microtiter polystyrene plates by ultraviolet irradiation. Both polysaccharide antigens could be coupled simultaneously to the same microtiter plate. The coated surface was used in indirect ELISA for the determination of serum antibodies from pigs infected with bacteria of the two *Salmonella* groups and from uninfected pigs. This ELISA proved itself by having a good long-term durability and a high degree of reproducibility, including low day-to-day variations and low interplate variations. Furthermore, the ELISA showed good specificity and sensitivity when data were compared with the optical density levels of a panel of pig sera as determined by a conventional ELISA on the basis of passive coating of the two *Salmonella* LPS antigens (the mix-ELISA). The covalent anthraquinone mix-ELISA shows promise as a stable and durable alternative to the existing conventional ELISA for serological surveillance of *Salmonella* infections in pigs.

Bacterial lipopolysaccharides (LPSs) are important serotype-defining antigens of gram-negative bacteria,¹⁷ and they are often, therefore, used as antigens in serological tests for the detection of serotype-specific antibodies.^{2,10} Lipopolysaccharide consists of the hydrophobic lipid A part and the hydrophilic polysaccharide (PS) part. The latter can be further subdivided into a constant core region roughly similar in all Enterobacteriaceae and the variable O-antigenic PS specific for the bacterial serotype.^{4,13} Lipopolysaccharide is anchored to the outer bacterial membrane by lipid A, whereas the highly immunoreactive PS part is exposed and able to interact with the immune system of the infected host.¹⁷

In conventional solid-phase immunoassays (e.g., ELISA) for detection of anti-LPS antibodies, purified LPS is passively adsorbed to the plastic surface by hydrophobic interactions between the lipid A and the plastic surface, leaving the antigenic PS exposed to the solvent and freely accessible to the antibodies. However, coating by passive adsorption is strongly dependent on the overall amphiphilicity of the LPS, being in turn dependent on the specific type of LPS, and because purified LPS is heterogeneous because of the

natural variability of the O-specific PSs, that may differ within a purified preparation of LPS.¹² With certain serotypes, this may lead to problems with reproducibility,¹⁰ and, especially in assays containing more than one antigen, competition for coating between the LPS antigens may occur, possibly reflecting differences in readiness of binding to the surface. Another drawback of using intact LPS as coating antigen is that lipid A may potentially be the cause of cross-reactions^{13,17} because this region is identical in most Enterobacteriaceae.

In the surveillance of *Salmonella* antibody levels in pigs, estimation of levels of anti-*Salmonella typhimurium* and *Salmonella infantis* antibodies in pig serum¹⁰ or muscle fluid¹¹ was shown to be a valuable means of screening large pig populations for *Salmonella* infections at the herd level.⁸ In the Danish pig production system, *S. typhimurium* O: 1, 4, 5, 12 is the dominating serotype found in naturally *Salmonella*-infected pigs (61% of 302 infected herds),¹ followed by smaller proportions of various serotypes.

Accordingly, an ELISA for broad screening for *Salmonella* antibodies, employing a mixture of passively coated LPS antigens from *S. typhimurium* and *S. choleraesuis*, was estimated to detect infections by at least 93% of all serovars occurring in *Salmonella* infections in Danish pigs.⁸ *Salmonella choleraesuis* LPS was used in this ELISA instead of *S. infantis* LPS because the latter was found to give irreproducible results.¹⁰

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Here, a novel technology for immobilizing bacterial PSs to microtiter plates is utilized to produce a covalently coated surface.⁵ *Salmonella typhimurium* and *S. choleraesuis* PSs were prepared from LPS and then conjugated to a photoreactive anthraquinone (AQ) derivative. This preparation, in turn, was attached covalently to the polystyrene surface by ultraviolet (UV) irradiation creating the diagnostic surface for the AQ mix-ELISA. The optical density (OD) levels obtained in the two assays were compared with the use of a panel of sera, previously characterized by the standard mix-ELISA with respect to levels of antibodies against *S. typhimurium* and *S. infantis* (O-types 1, 4, 5, 12, 6, and 7), to evaluate the possibility of using the AQ mix-ELISA in the same way as the standard mix-ELISA as a screening test for porcine *Salmonella* infections. The reproducibility of the AQ mix-ELISA was estimated with the same panel of sera as mentioned above.

Materials and methods

Pig sera and muscle fluid samples. Four *S. typhimurium*-positive, two *S. infantis*-positive, and one *Salmonella*-negative serum were used as references to calibrate the OD readings from each ELISA plate, exactly as described previously.¹⁰ Forty sera from pigs from multiplying herds were selected as a test serum panel to represent both high and low levels of antibodies as defined by the mix-ELISA. Muscle fluid samples obtained from each of 160 pigs at slaughter were tested in parallel in the AQ mix-ELISA and the mix-ELISA.

Bacterial strains. *Salmonella typhimurium* no. 3389-1/92 (O: 1, 4, 5, 12) and *S. choleraesuis* var. Kunzendorf no. 143 (O: 6, 7) were used for the preparation of LPS. The strains were grown aerobically at 37 C on solid growth medium containing agar^a supplemented with 5% bovine blood. Broth cultures were grown in Luria-Bertani (LB) broth⁷ in flasks overnight at 37 C with agitation (130 rpm). A 7-liter bioreactor^b containing 4 liters of LB broth was inoculated with 800 ml overnight broth culture. Incubation was done at 37 C, pH 7.2, pO₂ at 50%, agitation at 500 rpm. Foam was controlled by addition of silicone emulsion. Ninety minutes after inoculation, 400 ml 25% glucose was added. Five hours after inoculation, aeration was reduced to approximately 1 liter/min. Fermentation was carried out for 18 hr, and the culture was inactivated by adding formalin to a final concentration of 3% and continuing incubation at 200 rpm, 20 C without aeration. After 6 hr of incubation with formalin, the culture was checked for nonviability by overnight culture on agar^a as described above. If inactivation was complete, the inactivated culture was forwarded for further processing.

Extraction of LPS. The following operations were performed in a ventilated hood. Lipopolysaccharide was extracted from the formalin-killed cultures by hot aqueous phenol extraction.³ Briefly, the bacteria from 5 liters of culture were washed in 3 × 4 liters phosphate-buffered saline (PBS), followed by four washings in 5–10 volumes of acetone at –20 C, dried, and resuspended in 250–300 ml water. Lipopolysaccharide was then extracted from this suspension by

hot (65 C) 45% aqueous phenol by adding an equal volume of preheated 90% aqueous phenol to the suspension and keeping the mixture at 65–68 C for 10 min with gentle agitation. After cooling to 4 C followed by centrifugation for 30 min at 10,000 × *g*, the upper aqueous phase was carefully retrieved and dialyzed against water for at least 48 hr (with three shifts) at 4 C and subsequently freeze-dried.

Preparation of PS from LPS. Lipopolysaccharide (1–2 g) was dissolved in water (4 mg/ml) and subjected to mild acid hydrolysis in 1 M acetic acid at 90 C for 1 hr in sealed tubes.¹⁶ The liberated PS was extracted with 1.5 volumes of chloroform/methanol (2/1, v/v). The aqueous phase containing the PS was then dialyzed against three changes of water for 48 hr and subsequently freeze-dried. The organic phase containing the lipid A was discarded.

Conjugation of PSs to AQ and covalent attachment to microtiter plates. The mild hydrolysis of LPS cleaves the ketosidic bond between the 3-deoxy-D-manno-2-octulonic acid (KDO) residue of the PS and the *N*-acetyl glucosamine residue of the lipid A,¹⁶ thereby leaving the PS with at least one terminal KDO. The PS was then covalently coupled via its KDO residue to a photoreactive AQ derivative by a carbodiimide/*N*-hydroxy succinimide conjugation procedure, thereby forming a stable AQ-PS conjugate described elsewhere in detail.⁵ A mixture of *S. typhimurium* and *S. choleraesuis* AQ-PS conjugates dissolved in a buffered coating solution was immobilized covalently on microtiter plates^c by photocoupling (UV irradiation 300–400 nm, 20 min). After photocoupling, the plates were washed and dried (30 min at 37 C in a hot-air oven) and then stored in darkness at room temperature.

The plates were subsequently used in the AQ mix-ELISA as described below. Eight different batches of plates were used in this study, each batch of plates being produced on a different day.

ELISA procedure. The mix-ELISA was performed as described previously for serum¹⁰ and muscle fluid samples,¹¹ respectively. The AQ mix-ELISA was performed similarly except that the coating and blocking steps were omitted. Briefly, sera were diluted 1:400 (or 1:250) in phosphate-buffered saline (PBS), 0.05% Tween 20, and 1% (w/v) bovine serum albumin (BSA). Duplicates of 100 μl of each serum were applied and incubated for 1 hr at room temperature without agitation. The plates were then washed three times in PBS, and 0.05% Tween 20 and subsequently incubated with peroxidase-conjugated rabbit anti-swine immunoglobulins^d diluted 1:2,000 in PBS, 0.05% Tween 20, and 1% (w/v) BSA for 1 hr at room temperature. The plates were then washed as before, and 100 μl substrate (0.01% H₂O₂, 0.66 mg/ml 1,2-O-phenylene diamine dihydrochloride in 0.1 M citrate, pH 5) was added to each well and incubated 10–15 min. The reaction was stopped with 100 μl 0.5 M H₂SO₄, and the OD was read at 490 nm, subtracting 650 nm (background correction).

ELISA, calibration, and determination of cutoff values. The OD values obtained by photometrical readings were calibrated by the seven in-house reference sera spanning the range of measurements, each of them having normative OD values estimated by repetitive testing. The actual readings were transformed into OD% in the following way. The seven

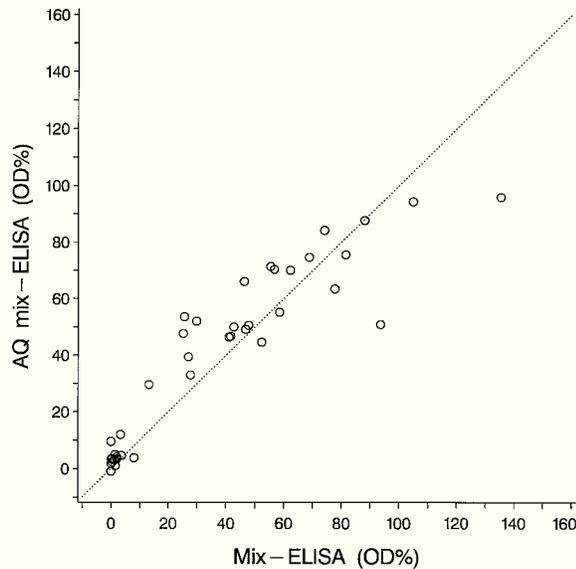


Figure 1. Correlation between the AQ mix-ELISA and the mix-ELISA with a panel of 40 serum samples obtained from multiplying pig herds. Each serum sample was tested 10 times during 1 month in the two ELISA systems. The values are averages of 10 rounds of testing in OD%. A line depicting $y = x$ is inserted.

references were included on all microtiter plates, and the calibration was made by linear regression analysis of the actual OD values of the references upon the normative OD values of the same references. The OD values of the samples were then converted into calibrated OD values by the regression equation obtained and further converted into OD% by dividing the calibrated OD value with the normative value of a given reference serum.

The classification of sera into positives and negatives was done as described previously⁸ with the experimental cutoff value at 10 OD%, which was defined previously on the basis of reactivities of sera from 25 *Salmonella*-negative pigs experimentally infected with *Yersinia enterocolitica* O:3 in the mix-ELISA.¹⁰ The monitoring cutoff at 40 OD%, above which it was possible to detect *Salmonella* in fecal samples, was derived from the Danish *Salmonella enterica* surveillance and control system.⁸

Design of experiments. The AQ mix-ELISA was tested and compared with the standard mix-ELISA in four different settings.

Experiment 1: Repeated testing over 10 days during 1 mo of 40 serum samples collected from Danish multiplying herds.

Experiment 2: One hundred sixty muscle fluid samples from pigs at slaughter tested once.

Experiment 3: Repeated testing on the same day of 40 test sera and seven reference sera on six AQ mix-ELISA plates.

Experiment 4: Extended repeated test of seven batches of AQ mix-ELISA plates. Plates were tested over three 3-wk periods. Each of the seven batches was tested four times distributed on three different days in each 3-wk period. The test serum panel and the seven reference sera were used in this test. From the start of the production of AQ mix-ELISA plates to the end of testing, a period of 3.5 mo elapsed.

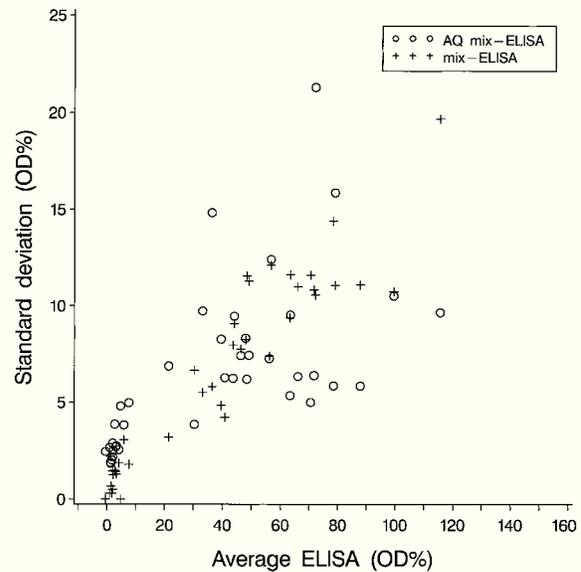


Figure 2. Standard deviations (OD%) in the AQ mix-ELISA and the mix-ELISA depicted against the average OD% of the AQ mix-ELISA and the mix-ELISA, obtained in the 10 repetitive tests of the panel of 40 serum samples from multiplying pig herds.

Statistical methods. The degree of association between OD values from the AQ mix-ELISA and the mix-ELISA was measured by Spearman's rank correlation coefficient¹⁵ because it was clearly inappropriate to assume normal distributions; *P*-values are given for the hypothesis of no association. Exact 95% confidence intervals for sensitivity and specificity of the AQ mix-ELISA relative to the mix-ELISA were calculated from the binomial distribution.⁹ The OD readings from the extended repeated test were analyzed by a mixed linear model⁶ after square-root transformation to achieve approximately equal variances. Estimated batch levels within the three periods of testing—standardized at an OD level of 1.0—were based on an analysis of the full data set comprising all periods, whereas estimated pooled reproducibilities (mean square errors) from plate replication for each batch and period were converted into prediction intervals for a single observation, centered also at an OD level of 1.0.

Results

Experiment 1. Anthraquinone mix-ELISA and mix-ELISA OD% of the 40 test sera are depicted in Fig. 1; values are averages of 10 rounds of testing. The association between the two sets of OD values was strong (rank correlation coefficient = 0.92; $P < 0.001$), and the OD values in the AQ mix-ELISA were generally somewhat larger except for the uppermost range of mix-ELISA values with reduced linearity between optical readings and antibody concentrations. Also, the standard deviations (SDs) of the 10 repetitive tests in both ELISA systems are depicted (Fig. 2) as a function of the average OD%. The SDs of the positive samples were generally lower in the AQ mix-

Table 1. Grouping of OD values (averages from 10 rounds of testing) of 40 test sera from multiplying pig herds into positive and negative samples with cutoff values at 10 OD% and 40 OD%, with estimated sensitivities and specificities (95% confidence intervals) of AQ mix-ELISA relative to mix-ELISA.

Mix-ELISA	AQ mix-ELISA	
	+	-
Cutoff at 10 OD%*		
+	25	0
-	1	14
Cutoff at 40 OD%†		
+	19	0
-	3	18

* Sensitivity = 25/25 = 100% (86–100%); specificity = 14/15 = 93% (68–100%).

† Sensitivity = 19/19 = 100% (82–100%); specificity = 18/21 = 86% (64–97%).

ELISA, whereas the SDs of the negative samples were slightly higher but not exceeding 5 OD%. In both ELISAs, most samples had SDs below 15 OD%.

With the experimental and monitoring cutoffs at 10 OD% and 40 OD%, respectively, the samples were classified as positive or negative in both ELISA systems on the basis of the average OD% (Table 1). At both cutoffs, all mix-ELISA-positive samples were also positive in the AQ mix-ELISA. At the cutoff at 10 OD%, one sample that was negative in the mix-ELISA was positive in the AQ mix-ELISA, and at the cutoff at 40 OD%, three of the samples that were negative in the mix-ELISA were positive in the AQ mix-ELISA. Thus, the estimated sensitivity of the AQ mix-ELISA relative to the mix-ELISA was 100% at both cutoff levels, and the estimated specificities were 93% and 86%, respectively.

Experiment 2. The OD% of muscle fluid samples tested simultaneously in the AQ mix-ELISA and the mix-ELISA are shown in Fig. 3 (rank correlation coefficient = 0.86; $P < 0.001$). The maximal OD% difference between the two systems was 13 OD%. At the monitoring cutoff (40 OD%), all the muscle fluid samples classified as negative in the mix-ELISA were also negative in the AQ mix-ELISA. Three samples that were positive in the mix-ELISA were negative in the AQ mix-ELISA.

Experiment 3. Interplate variations for measurements on the same day, defined as the SD between values obtained from different plates for each of the 47 sera, were related to the size of the OD values (Fig. 4). In this case, the performance of the AQ mix-ELISA in terms of interplate variation of the plates was calculated from the noncalibrated OD values because calibration of the data might reduce any occurring heterogeneity of the plates. The average SD of the sero-

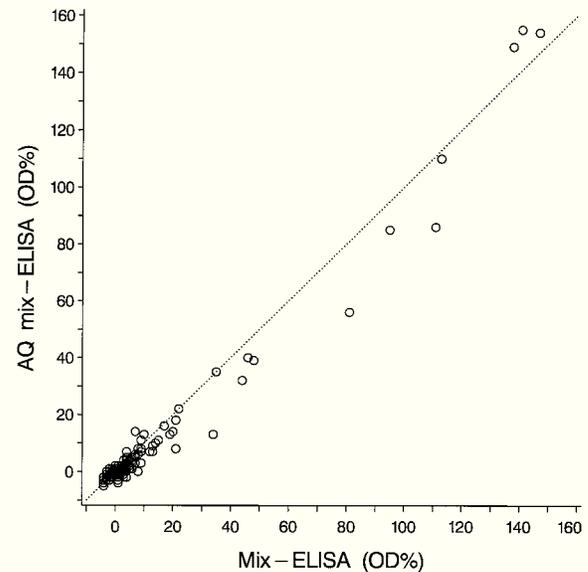


Figure 3. Correlation between the AQ mix-ELISA and the mix-ELISA with 160 muscle fluid samples taken at slaughter. The AQ mix-ELISA and the mix-ELISA were run simultaneously in the laboratory. A line depicting $y = x$ is inserted.

positive sera (having OD% > 10 in the mix-ELISA) was 0.11 OD, and the average SD of the seronegative sera was 0.02 OD. In a test performed over 3 days with two plates per day, similar SDs were obtained (not shown).

Experiment 4. The estimated OD levels standardized at OD = 1.0 varied between 0.82 and 1.23 for the seven different batches and the three different periods tested (see Table 2). The estimates were pooled

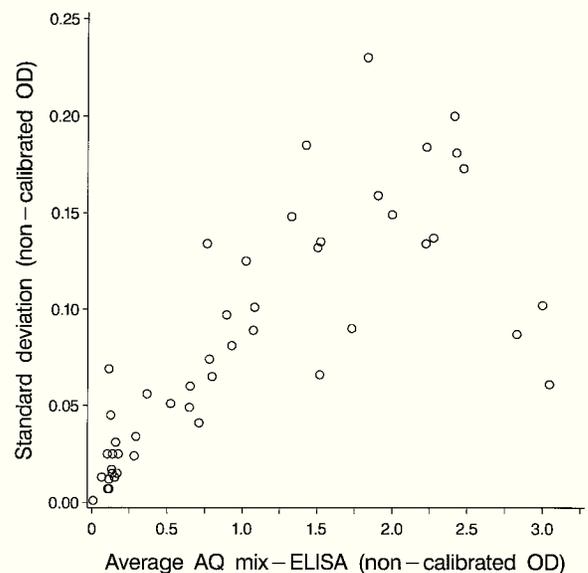


Figure 4. The interplate variation expressed as sample SDs (OD values) of the panel of 40 serum samples from multiplying pig herds as a function of the average OD values of the AQ mix-ELISA and the mix-ELISA.

Table 2. Estimated OD levels of the test and the reference sera normalized at OD = 1.0 and compared between batches a–g and periods I–III on the basis of noncalibrated OD readings from four runs of plates from each batch in each period.

Batch	OD = 1.0 normalized estimates		
	Period I	Period II	Period III
a	0.82	1.00	1.03
b	0.91	1.01	0.95
c	0.94	1.07	1.10
d	0.87	1.03	1.00
e	0.88	1.00	0.99
f	1.08	1.23	1.23
g	0.87	1.03	1.02
Average	0.91	1.05	1.05

for all sera and corrected for day-to-day and interplate variations. The average estimates of the three periods of testing were 0.91, 1.05, and 1.05, respectively. Even though some of the estimates differed significantly (the standard error of mean is 0.06 at the 5% level), no general trend from batch to batch or from period to period could be observed, indicating that the observed variations were not due to irregularities in the coating or loss of antigen over time.

The 90% prediction intervals centered around OD = 1.0 varied between 0.15 and 0.73 (Table 3). This value is a measure of the reproducibility between two plates run on the same day. Some values were significantly different (i.e., their ratio exceeded 1.3), but again no general trend could be observed.

Discussion

Lipopolysaccharide is an amphipathic molecule that is believed to bind to plastic surfaces in an equilibrium between free LPS, plastic-bound LPS, and LPS-LPS complexes (micelles). The overall tendency of LPS to coat to a given surface is thus dependent on exact amphiphilicity of the molecule.¹⁴ When attempting to coat more than one type of antigen at the same time, it was expected that a solution of different PSs (O-antigens) derivatized with AQ could be coupled in a more controlled and noncompetitive way than a mixture of different, unmodified LPS types having different amphiphilicities. Another concern was to avoid lipid A cross-reactivity as well as to increase the reproducibility of coating compared with coating with intact LPS. *Salmonella* PSs derived from purified *Salmonella* LPS can be derivatized with the photoreactive compound AQ and subsequently immobilized on polystyrene microtiter plates by UV irradiation.⁵ With this technology, an antigenic surface was developed that formed the basis of an indirect ELISA for detection of pig serum antibodies against *Salmonella* LPS antigens. To test pig sera for the presence of antibodies against the two most common serovars of *Salmonella* in Dan-

Table 3. Lengths of 90% prediction intervals for a single observation centered around OD = 1.0, calculated from two same-day runs of plates from each batch in each period.

Batch	Length of 90% prediction intervals*		
	Period I	Period II	Period III
a	0.25	0.23	0.25
b	0.31	0.23	0.42
c	0.28	0.15	0.28
d	0.28	0.38	0.55
e	0.73	0.23	0.41
f	0.37	0.46	0.33
g	0.40	0.22	0.45

* The interval within which, on the average, 90% of values will lie; for example, an interval length of 0.25 corresponds to an interval of 0.88–1.13.

ish pig herds, the coating was done with a mixture of LPS antigens from *S. typhimurium* and *S. choleraesuis* LPS analogously to the previously described mix-ELISA.¹⁰ The coating layer thus consisted of a mixture of photocoupled carbohydrates derived from LPS from these two types of *Salmonella*, resulting in a surface covalently coupled with carbohydrate antigenic factors 1, 4, 5, 6, 7, and 12. Assay conditions were similar to the standard mix-ELISA except the blocking, which was omitted because it was found to have no influence on the assay.

At both 10 and 40% cutoff levels, high correlations between the AQ mix-ELISA and the mix-ELISA were obtained, with a sensitivity of 100% and specificities of 93% and 86%, respectively, at the two cutoff levels. These correlations indicate that, for classification purposes,⁸ the AQ mix-ELISA can be used with the same cutoff levels as the mix-ELISA. The high correlation between mix- and AQ mix-ELISAs indicates that the antigenicity of the PSs upon delipidation, conjugation to AQ, and photocoupling to the polystyrene plates seems to be fully conserved.

The high correlation between the two assays was also seen with muscle fluid samples; in this case, no false positives were seen but a few false-negative samples were obtained by the AQ mix-ELISA. Because the maximum difference between the two tests with muscle fluid samples was only 13 OD%, this small decrease in sensitivity likely can be remedied by adjusting the cutoff of the AQ mix-ELISA. The interplate variations of the AQ mix-ELISA plates were very low, as shown in Fig. 4, indicating a very homogeneous coating of the microtiter plates.

The estimates obtained from the extended repeated test were more similar between batches than between periods, indicating that plates had been reproducibly coated from batch to batch and that the practical handling of the plates caused larger variations. Although some of the estimates differed significantly, no general

trend and no indication of any time-dependent change (e.g., decrease) in the OD levels were obtained over time. Also, the OD levels obtained in the three periods were similar to OD levels obtained on these plates just after coating (not shown), and thus the coating was stable, without any sign of deterioration for at least 3.5 months when the plates were stored dry at room temperature. The corresponding LPS-coated mix-ELISA plates have a durability of approximately 14 days stored covered at 4 °C containing the coating solution (N. Feld, Danish Veterinary Lab., Denmark, personal communication).

When the length of the 90% prediction intervals derived from same-day tests of two plates from each batch in each of the three periods was determined, significant variations were again observed with no apparent trend over time or between batches. As with the estimates, it can be concluded that reproducibilities of the plates do not deteriorate over time and that the differences may rather be due to irregularities in the handling of the plates. Further investigations of the performance and the durability of the AQ mix-ELISA will be carried out in order to evaluate its usefulness for large-scale routine screening of pig herds with muscle fluid samples.¹¹

The excellent reproducibility of the AQ mix-ELISA combined with the stability and easy handling of the plates make the AQ mix-ELISA highly suitable for screening programs where a uniform long-term assay performance is required. The fact that the plates can be manufactured centrally, stored for lengthy periods, and distributed by ordinary mail also makes the test useful for standardization and for validation of assays between different laboratories.

The specific combination of antigens used in this study was tailored for *Salmonella* screening of pig herds in Denmark, but the technique is easily applied to other types of LPS-derived PSs because the coupling of AQ is dependent only on sugars typically present in the core, which are strongly conserved among all gram-negative bacteria.⁴ The method of coupling a mixture of carbohydrate antigens representing different bacterial strains is of general use for analyzing samples in one step for infections with more than one bacterial strain.

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Sources and manufacturers

- a. Columbia agar, Oxoid, Unipath Ltd., Basingstoke, UK.
- b. Bio-reactor, MBR Bio Reactor AG, Wetzikon, Schweiz.
- c. Polysorp ELISA plates, Nalge NUNC International, Roskilde, Denmark.
- d. Conjugate P0164, DAKO A/S, Glostrup, Denmark.

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