VALIDATION OF A SURFACE PLASMON RESONANCE BASED ASSAY TO DETECT *SALMONELLA* ANTIBODIES IN SERUM OF PIGS

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Abstract The aim of this study was to develop and validate a novel serological test employing surface plasmon resonance (SPR), that was designed for use on the Biacore Q apparatus to detect antibodies against *Salmonella Spp* in pig sera. *Salmonella Spp* antigens (sero-groups B and D) were immobilized onto CM-5 sensor chips (Biacore). Test conditions and apparatus adjustments were optimized. Subsequently, validation was done using positive and negative ("in house") reference sera as well as 990 field sera collected at slaughter. A set of 990 sera originating from finishing pigs were tested in the SPR assay and ELISA. Overall agreement between the two tests amounted to 92%. From these results it is concluded that the SPR assay in conjunction with the Biacore Q apparatus is suitable for screening slaughter pigs against *Salmonella* in a surveillance program.

Introduction Surface plasmon resonance (SPR) spectroscopy is a label free technology for monitoring biomolecular interactions in real time (Turbadar, 1959, Kretschmann and Raether, 1968). SPR occurs at the surface of a gold film that is adhered to a glass plate (the sensor chip). When incident light is directed to the gold film it causes electrons on the surface of the gold film to move in waves (surface plasmons). A prerequisite for this to occur is that the incident light reaches the gold film at a certain angle (the resonance or SPR angle). The angle of the reflected light changes when the refractive index at the sensor surface changes. Since the relation between the two is linear, the change in resonance angle can be used to measure changes in refractive index caused by binding of analyte to a covalently immobilized ligand on the sensor chip surface. Hence the angle value of the reflected light is changed and measured in relative resonance units (rRU). Events are displayed in real time by a sensorgram on the screen of the computer that instructs the Biacore Q apparatus.

In Europe a number of countries have started serological surveillance programs for *Salmonella* infections in slaughter pigs. Serum or meat drip samples of slaughtered pigs are generally tested by ELISA of which several are commercially available. The Netherlands initiated a sero-surveillance program for finishing pigs as of January 2005. This can be done by testing pig sera by ELISA for the presence of antibodies against *Salmonella Spp* in sera and/or meat drip (Nielsen *et al*, 1995, Van der Heijden *et al*, 1995, Gabert *et al*, 1999, Proux *et al*, 2000). In this paper we describe the validation of a novel test, based on SPR that detects porcine *Salmonella* antibodies directed to sero-groups B and D. Validation of the Salmonella SPR assay has been focused on test characteristics that determine the validity (specificity and sensitivity). In addition, precision (repeatability and reproducibility) is discussed. Validation tests have been performed using the Biacore Q apparatus.

Materials and Methods A set of 990 field sera was collected (during 2003/4) from finishing pigs brought at slaughter. The sera originated from 57 pig holdings with a sample size ranging from 14 to 22. All sera were tested in a 1/5 dilution made in HBS-EP (Biacore).

With each test run two reference sera were included (Wright *et al*, 1993). Reference serum 1 is a strong positive serum expected to display approximately 800 rRUs (relative response units). Reference serum 2 is a negative serum that is expected to result in approximately 100 rRUs (background signal). The reference sera were tested at the start, middle and end of each test run. The mean results of the references were used to calculate test results to compensate for wear and tear of the sensor surface.

Sensor chips were made according to a standard protocol. Briefly, lipopolysaccharides (LPS) originating from cultures of *Salmonella* Typhimurium (sero-group B) and *S. enteriditis* (sero-group D) were extracted and purified. Subsequently, a mixture of LPS-B and D, with predetermined concentrations of the individual constituents, was immobilized onto carboxy-methylated dextran (CM)-

5 chips using a standard aldehyd coupling as described in the manual of Biacore (anonymous, 2003). A sensor chip consists of 4 flow cells and in each flow cell approximately 500 sera can be tested.

The testing of a serum sample in the Biacore Q apparatus was a cycle consisting of: (i) injection of run buffer in the flow system (base line determination), (ii) sample injection (2'), (iii) injection of run buffer, (iiii) regeneration (removal of bound analyte with a flow rate of 100Ìl per minute for 20") of the sensor surface.

The rRUs are considered to be absolute measurements (raw data), which have to be converted to normalized data (Jacobson, 1996). To account for variability of test conditions, results are expressed as a function of the reactivity of one or more reference samples that are included in each test run (1). For this reason test results of the Salmonella SPR assay are converted to percentage positivity (PP) values calculated according to the formula:

PP = (rRU sample minus rRU ref. serum 2 / rRU ref. serum 1 minus rRU reference 2) x 100

The PP value at which sera are considered negative or positive in the SPR assay (cut off value) was determined using the test results of the set of 990 sera by placing the cut off value at the point of maximal overall agreement with the ELISA.

In the absence of a well defined reference serum panel for *Salmonella* (allowing the calculation of the absolute SE and SP) the relative SE (sensitivity) and SP (specificity) of the SPR assay were determined using a commercially available ELISA (Idexx), according to the manufacturer's instructions. The manual of the ELISA gives several cut off values that can be used. We selected the cut off value (OD% 40) that was recommended for testing field sera. The 990 sera originating from finishing pigs were tested in the Salmonella SPR assay and the ELISA.

Repeatability and reproducibility of the SPR assay was determined by testing the 2 SPR reference sera and a weak positive (WP) and a strong positive (StP) serum. To determine repeatability, each of the sera were tested 24 times in one flow cell of a routinely produced sensor chip in one test run of 96 samples. The CV% (coefficient of variation) was calculated using the raw data (relative resonance units –rRU-) and the normalized raw data (percentage positivity –PP-). To determine reproducibility test results have been gathered that were produced in a one month period (10-09-04/13-10-04). Tests were executed on 4 different Biacore Q apparatuses which were operated by two technicians. In total all flow cells of 5 chips (BD01, 02, 04, 05 and 06) were used to their maximum capacity (approximately 500 sera per flow cell). Reference sera were tested in triplicate per routine test run that consisted of approximately 130 sera.

Results Results of the SPR assay and ELISA are summarized in table 1. In addition, the optimal cut off for the SPR assay was determined at a value of 30 PP resulting in the highest possible overall agreement of 92% and Cohen's kappa of 0,74.

Boxes B and C (table 1) contain the number of sera with conflicting test results. The ELISA detects more positive sera than the *Salmonella* SPR assay which results in a lower over all agreement. Consequently, the inferred relative SE and SP of the *Salmonella* SPR assay amounted to lower percentages (83% and 94%, respectively). However, from table 2 it can be seen that on farm level (the intended use of the SPR assay) results vary only slightly.

The variation of SPR test results measured within one flow cell of a sensor chip differed minimally resulting in a sufficiently low CV% using the raw data or normalized data (table 3) for the calculation. All CV% of the reference samples were well below 5% indicating a well controlled test process.

The CV% for reproducibility of the *Salmonella* SPR assay was well below 20 when using raw data for the calculation. Moreover, usual practice is to use the normalized data for this calculation. The PP values of the WP and StP sera displayed a CV% well below 10. Results obtained with the *Salmonella* SPR assay indicate a well controlled process with reference to the reproducibility.

Idexx ELISA	SPR assay		
	Pos	Neg	Total
Pos	(A) 144	(B) 48	192
Neg	(C) 30	(D) 768	798
Total	174	816	990

Table 1: Test results of 990 pig sera obtained with the ELISA and *Salmonella* SPR assay.

Discussion Preferably, newly developed tests are validated using well defined serum panels. Validation of tests for the detection of antibodies directed against *Salmonella Spp* is hampered by the absence of such a panel. For this

Cut off values		40	30
Farms	n	ELISA	SPR
1	20	4	3
2	12	0	0
3	19	1	0
4	19	1	0
5	20	0	0
6	21	16	17
7	22	2	0
8	20	0	2
9	22	17	16
10	18	13	13
11	18	0	1

Table 2: Selection of test results on farm level tested by the Idexx ELISA and the *Salmonella* SPR assay displaying the number of positive sera per test and farm

reason, we chose to compare the *Salmonella* SPR assay performance with a commercially available ELISA. The overall agreement of the Salmonella SPR assay compared to the Idexx ELISA (92%) can be considered more than sufficient to guarantee reliable test results. Moreover, the comparison has been made with sera originating from individuals while the intended use of the *Salmonella* SPR assay is at herd level. When the sera originating from individual herds are grouped together, then the results of both tests do not differ in the sense that e.g. one herd is completely negative and another test classifies many sera of that herd as positive. In addition, the relative sensitivity (SE) of the *Salmonella* SPR assay with both ELISA's is 83% coupled to a specificity (SP) of ~93%. Again, these figures have been calculated on an individual basis. Considering the results displayed in table 2 it may be expected that calculated on herd level the relative SE and SP of the both tests is highly comparable. Unfortunately, we were not able to calculate the SE and SP on herd level since we did not have a sufficient number of sera per pig farm for a valid calculation.

It must be noted that the ELISA manufacturer claims detection of *Salmonella* sero-types B, C, and D. Since the *Salmonella* SPR is based on sensor chips with immobilized sero-type B and D antigen it may add to slight differences between the ELISA and the *Salmonella* SPR assay. However, we found that the serological prevalence of *Salmonella* Spp is predominantly determined by sero-type B (data not shown). The determination of the precision of the *Salmonella* SPR assay as portrayed by repeatability and reproducibility resulted in highly sufficient coefficient of variation (CV) percentages. This means that the short- and long term test performance (that is, reliable test results!) is warranted despite the fact that conditions (newly produced chips, different batches of active components etc.) vary.

Conclusions The developed SPR assay to detect *Salmonella* antibodies in serum of slaughter pigs was highly comparable with a commercially available ELISA (Idexx). Therefore it can be concluded that the developed SPR assay is suitable for use in serological surveillance programs for *Salmonella* in slaughter pigs.

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N = 96	Ref 1	Ref 2*	Ref 3	Ref 4
Mean	100	0	38	379
Std	2,38	0	1,14	11,49
CV%	2,47	0	3,03	3,03

Table 3: Repeatability statistics of the four reference samples with test results based on PP values. *Negative reference sample and the PP value is per definition zero.