Porcine reproductive and respiratory syndrome antibody detection on filter discs

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Summary
Two enzyme-linked immunosorbent assay (ELISA) kits were evaluated for detection of porcine reproductive and respiratory syndrome (PRRS) antibodies in pooled sera and filter discs (FDs). Elution and incubation procedures and positive thresholds for both ELISAs were determined using FDs collected from sixty non-infected pigs and five pigs with low PRRS-antibody titres. Eighty paired samples (serum/FD) from infected pigs were titrated using both ELISAs. The authors thus showed that five sera or five FDs could be pooled in one test without significant loss of sensitivity. Compared to individual sera, method sensitivity was found to be 79% and specificity 97.5%, based on data from 200 pools of FDs collected on 15 PRRS-infected farms and 120 pools collected on 71 non-infected farms. To balance loss of sensitivity, two pools of five samples from sows and one pool of five samples from finishing pigs can be tested as an alternative to seven and five single sera, respectively.

Introduction

Porcine reproductive and respiratory syndrome (PRRS) is a contagious viral disease of swine that was first observed in North America in 1987-1988 (2, 5) and in Europe in 1990 (7). The disease is described in section X under 'Other diseases of importance in international trade' of the OIE (World organisation for animal health) *Manual of standards for diagnostic tests and vaccines* (9). The disease is mainly characterised by reproductive failure (abortion, stillbirth, birth of weak piglets and piglet death) and respiratory distress of piglets and growing pigs (flu-like syndrome including recurrent fever, inappetence, signs of pneumonia and increased mortality related to the development of secondary bacterial infections). The source of infection is an arterivirus (8), recently classified in the order of Nidovirales, family Arteriviridae, genus *Arterivirus* (3). Following an epidemic phase during the nineties, the disease has now become endemic in many swine-producing countries.

A general control programme was implemented in parts of France and prevalence of infection has been maintained below 2% (6). This control programme involved more than 2,000 farms and relied primarily on serological monitoring of herds with an enzyme-linked immunosorbent assay (ELISA) developed by Albina *et al.* (1). Enzyme-linked immunosorbent assay kits which have been commercially available since 1995 were then used for routine surveillance. To date, all diagnostic investigations have been based on the testing of individual sera. The purpose of this study was to adapt pre-existing commercial ELISA kits for the detection of PRRS virus antibodies in pooled

sera or blood-soaked filter discs (FDs). The general objective was to reduce the costs of serological diagnosis and thus render final disease eradication by partial or total farm depopulation financially acceptable. Such diagnosis on pooled samples might also encourage the monitoring of PRRS in other regions that cannot afford large-scale serological surveys on single sera. This paper reports a method for testing pooled sera or FDs and compares the sensitivity and specificity of the technique with those of the test performed on individual sera.

Material and methods

Sampling procedure

Sera were obtained by separation of blood clots after venipuncture. The samples were stored at –20°C until required for use. Blood droplets obtained by small skin scarifications of the ear or tail were collected on filter paper. Small filter discs with a radius of 5 mm were subsequently punched out of the filter paper and eluted for various lengths of time by gently stirring in 100 µl of different buffers. The resulting supernatants were collected and stored at –20°C until required for use.

Samples

In this study, 'negative pigs' refers to animals that were found to be negative for PRRS serum antibodies, belonging to farms never having experienced PRRS and regularly controlled as negative for PRRS antibodies by random sampling of the pigs. 'Positive pigs' refers to animals with PRRS antibodies in their serum from farms with a history of PRRS infection (disease and/or virus circulation confirmed by laboratory tests). Ten paired samples (one serum and one FD) collected from the same pig at the same time were tested to assess antibody detection using FDs. Five of these ten paired samples were obtained from negative pigs and the other five from positive pigs. Filter discs from 60 to 100 negative pigs and from five low-positive pigs were included in the study to define the positive threshold. The low-positive pigs were selected according to antibody titres in an immunoperoxidase monolayer assay (IPMA). Serum antibody titres of these animals were lower than 1/200.

Paired samples (serum/FD) from 60 to 80 pigs on 12 different farms infected for at least six months were titrated using an ELISA to determine distribution and average antibody titres. Samples were diluted in the serum or supernatant of eluted FDs from a negative pig bled at the slaughterhouse. Two hundred pools of five samples collected from 15 distinct farms that had been infected for more than six months were used to determine the sensitivity of the method on pooled sera or FDs compared to single sera. Similarly, 120 pools of FDs collected from 71 distinct uninfected farms were tested to determine method specificity. Positive samples were prepared for an inter-laboratory assay by pooling samples from one negative and one positive pig which had been bled at the slaughterhouse. One litre of serum and 200 FDs were prepared from each of these animals.

Enzyme-linked immunosorbent assay

Two commercial ELISA kits, referred to as Kit A and Kit B, were evaluated in the study with tests being performed on sera according to the instructions of the manufacturers. Different incubation times and temperatures were assessed for the eluted FD supernatants. Where necessary, additional tests using other diluents for FD elution were applied or kit composition was modified to improve performance.

The serum antibody titres of the test pigs were determined by IPMA (11).

Inter-laboratory comparison test

Ten pools of five FDs were prepared for an inter-laboratory comparison test. Filter discs were collected from one positive and one negative pig and pooled at different ratios : 1 (+) with 4 (–), 2 (+) with 3 (–), 3 (+) with 2 (–), 4 (+) with 1 (–). A pool with 5 (+) and one with 5 (–) was also included, as well as two replicates of the (2+/3–) and (3+/2–) pools. The samples were coded and sent to eight regional laboratories for testing according to the above-defined ELISA protocol.

Data analysis

The data were analysed essentially as described by Eloit *et al.* (4). The antibody titres of the sera and FDs were determined with each kit. The titres obtained were then plotted on a histogram showing sample distribution according to titre. The probability of obtaining a sample with a titre higher than or equal to 1, 2, 4, 8 or more, was then determined. The probability of a pool of N samples containing one positive sample to be detected positive with the ELISA corresponded to the probability that this positive sample had an antibody titre higher than or equal to N (the size of the pool or the dilution factor). This latter probability was calculated from the previously determined titre distribution. Accordingly, the probability for a pool of N samples containing n positive samples to be detected positive with the ELISA was the probability that these n positive samples had a mean antibody titre higher than or equal to N/n (the dilution factor). Therefore, the probability of detecting a pool with ($n/N = X$)% of positive samples corresponded to the probability that these X% positive samples had a mean antibody titre higher than or equal to $N/n = 1/X$%.

The probabilities of pool detection (P) according to the percentage of positive samples in the pool were calculated from the previously determined titre distribution using PC software. Pool size was then determined based on a compromise between detection probabilities (P), that should be as high as possible, and the practical and economical aspects of using pools instead of single sera. The authors decided that a minimum detection probability P of 40%-50% should be obtained when only one positive sample was present in the pool. The pool size required to obtain 40%-50% detection when there was only one positive

sample in the pool was then determined. The percentage X of positive samples required in a pool was back-calculated for a probability of detection ranging from 40% to 50%. Considering that one positive sample should be detected in the pool tested, then X should be $(1/PS) \times 100$ where PS defines pool size. In other words, pool size can be defined as $100/X$.

Once pool size had been established, the number of pools which needed to be tested on a farm to obtain the same sensitivity as with single sera was determined. In France, PRRS infection within a farm is usually confirmed by laboratory tests on seven sera collected from sows and/or five sera from finishing pigs. This sampling strategy ensures detection of 80% of infected farms (data not shown). The sensitivity of pool testing for PRRS diagnosis was then evaluated in comparison with single sera. If the sensitivity for one pool tested on a farm compared to the single sera included in the pool was S, then the sensitivity for n pools, Sn, became $100 - (100 - S)^n$. S was determined by laboratory testing, then n was calculated for $Sn = 80\%$.

Statistical analysis of the sensitivity, specificity and probability of detection of positive samples was performed at the 5% confidence level as described by Eloit *et al.* (4).

Results

Feasibility of the assay

The best results with Kit A and FDs were obtained when blood was first eluted from FDs in the diluent provided by the manufacturer for 2 h at room temperature under gentle stirring, followed by incubation of the supernatants with the ELISA antigen-coated plate overnight at 4°C. Kit B, based on the use of a single coated antigen (i.e. no mock antigen), gave false positive results, due to background reactions with some eluted blood. A new ELISA kit, including a mock antigen, was subsequently designed by the same manufacturer. With this new test, blood was eluted in the diluent provided by the manufacturer for 2 h at room temperature under gentle stirring. The sample was then diluted 1 in 5 in the diluent from the manufacturer and incubated with the ELISA antigen-coated plate overnight at 4°C.

Determination of the positive threshold for eluted filter discs

The results of the ELISA tests on samples eluted from FDs from 60 to 100 negative and five low-positive pigs are shown in Figure 1. The negative samples followed normal distributions. The positive threshold could therefore be calculated as the average result of the negative samples plus three standard deviations. Considering the normal distribution of these negative samples, 99.9% of all negative samples will give an ELISA score below this average plus three standard deviations.

With Kit A, the negative samples yielded an average sample/positive control ratio (S/P) value of 0.024 +/– 0.083. With Kit B, they gave an average percentage of positive control optical density (POD) of 6 +/– 6. The calculated positive threshold was therefore $S/P = 0.4$ for Kit A and POD = 30% for Kit B. With these thresholds, all but one of the low-positive pigs were detected as positive with the ELISA from Kit B (Fig. 1).

Determination of antibody titres in filter discs

About 60 to 80 paired samples (serum/FD) were titrated using the two ELISAs. Two-fold serial dilutions of these paired samples were made in the serum of a negative pig and in the supernatant of FDs collected from the same negative pig, respectively. Table I gives the average titres obtained with each kit and Figure 2 shows the distribution of these titres. The average titres and their distributions were very similar both for the serum and FDs on the one hand, and for the two kits, on the other hand. These distributions were used to determine the probabilities of detecting as positive, pools of sera or FDs containing a certain number of positive samples. First, the probability of a sample having a titre of 1, 2, 4… was calculated. Secondly, P, the probability of detecting N pooled samples containing n positive samples was determined as indicated above in 'Material and methods'. The results are reported in Figure 3.

The value P was shown to fall below 50% when X was less than 20% (less than 20% of the samples within the pool were positive). However, this probability rose to 80% when 40% of the samples were positive, i.e. $X = 40\%$. For both kits, a minimum detection probability of 40%-50% was determined for pools containing more than 20% positive samples, i.e. $X = 20\%$ (Fig. 3). Thus, pool size, PS, determined as described in 'Material and methods', was found to be $100/20 = 5$. For a pool of five samples, one and two positive samples in the pool gave 50% and 80% probability of detection, respectively. Different situations with a pool of five samples are represented in Table II. This Table shows that both kits behave in essentially the same manner.

Determination of method sensitivity and specificity for pools of sera or filter discs

Method sensitivity and specificity were determined using Kit A. Since the previous section showed that both kits yielded similar results, the determination was not repeated with Kit B. Two hundred pools of sera and FDs from 15 infected farms were used. Individual sera from pigs included in these pools (serum or FDs) were also tested by ELISA. The sensitivity and specificity of PRRS antibody detection in pools were compared to the results obtained with corresponding individual sera. The results are reported in Table III. Sensitivity of the pools compared to individual sera was found to be intermediate. The minimal sensitivity of the pools was 71% compared to single

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a) Kit A

b) Kit B
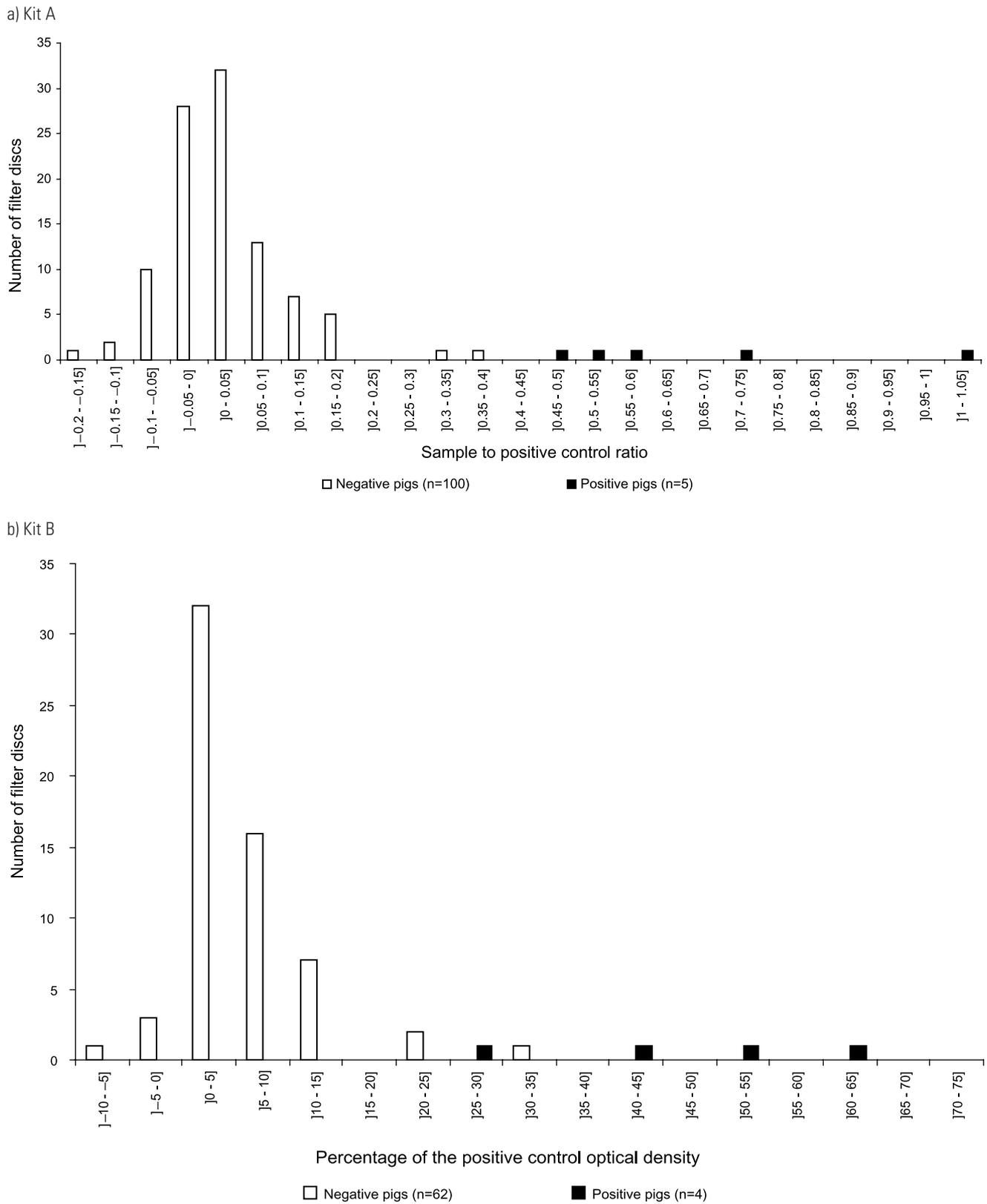
Fig. 1
Distribution of the results obtained with two enzyme-linked immunosorbent assays on 80 negative (open bars) and 5 low-positive pigs (full bars)
With Kit A, results were expressed as sample to positive control (S/P) ratios corresponding to the optical density (OD) of tested samples over the OD of the positive control. With Kit B, results were expressed as percentages of the positive control optical density (% POD). For both kits, ODs in the mock antigen wells were deduced from the ODs in the antigen wells

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Table I
Average porcine respiratory and reproductive syndrome (PRRS) antibody titres obtained with two commercial kits from paired samples (serum/filter discs) collected from twelve PRRS-infected farms
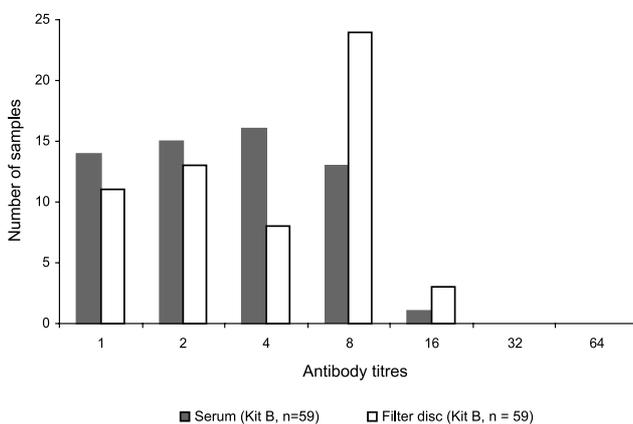
| | ELISA kit | |
	Kit A	Kit B
Serum	3.3 (n = 78)	2.9 (n = 59)
Filter disc	3.3 (n = 78)	3.6 (n = 59)

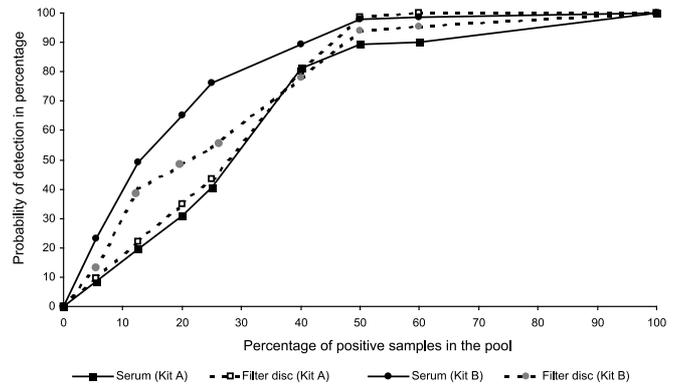ELISA: enzyme-linked immunosorbent assay

a) Kit A
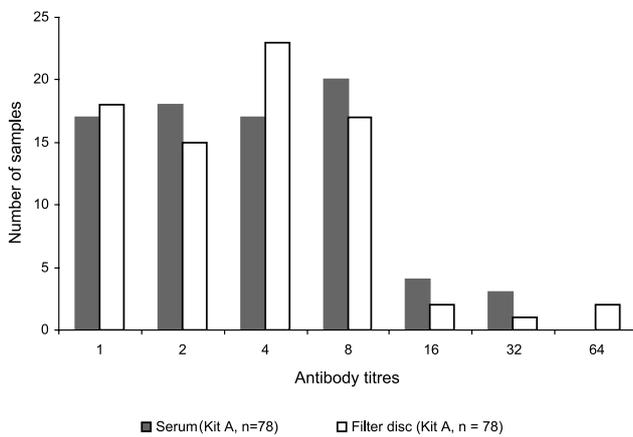
b) Kit B

Fig. 2
Distribution of serum and filter disc antibody titres determined by enzyme-linked immunosorbent assays in paired samples collected from 12 porcine respiratory and reproductive syndrome-infected farms

Fig. 3
Probability of detecting pools of samples containing different percentages of positive samples
Probabilities were determined from the distribution of titres shown in Figure 2. The figure shows that when the percentage of positive samples in the pools increased from 0 to 100, the probability of detecting positive pools increased exponentially

Table II
Probability, as a percentage, of detecting as positive pools of five samples containing one to five positive samples

| Number of positive samples | ELISA kit | | | |
| | Kit A | | Kit B | |
	Pools of sera	Pools of filter discs	Pools of sera	Pools of filter discs
1	45	50	65	49
2	68	77	89	79
3	84	88	98	94
4	91	95	99	97
5	100	100	100	100

ELISA : enzyme-linked immunosorbent assay

Table III
Sensitivity and specificity of the analysis on pooled samples compared to single sera
This study was carried out with enzyme-linked immunosorbent assay (ELISA) Kit A on 200 pools of five samples from fifteen porcine respiratory and reproductive syndrome (PRRS)-infected farms. The specificity of pool detection reflects the capacity of the ELISA to score negative pools containing only samples from pigs which have no detectable PRRS antibodies in their serum

Test performed	Pools of five sera	Pools of five filter discs
Sensitivity/single sera	71%	79%
Specificity/single sera	100%	86%

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sera. The specificity of the FD pools was also lower than with single sera. However, this decrease in specificity was considered with caution since all FDs had been sampled from infected farms and some might have been true positives, although the corresponding serum was negative.

Both ELISA kits were therefore further evaluated on 120 pools of five FDs collected from 71 non-infected farms to better assess the specificity of detection on pools. Specificity was then found to be significantly higher than 97.5% (p = 0.05). To validate the method developed for routine testing of FD pools, an inter-laboratory comparison test was designed. Eight regional veterinary diagnostic laboratories received twelve samples: ten pools of five FDs, one positive serum and one positive FD. The latter two samples had to be titrated by the laboratories. Table IV shows the results obtained with both ELISA kits. All the laboratories obtained satisfactory results with both kits, except for one laboratory (No. 8) with Kit A, thus illustrating the suitability of the method for routine application. Four

laboratories also detected pool 9 with Kit A whereas the others did not. This demonstrates that pool 9, containing only one positive FD, was close to the detection limit of Kit A and was subsequently detected by only half of the laboratories.

In France, PRRS serological diagnosis relies on tests on about 12 single sera (seven sows and five finishing pigs). Since method sensitivity was 71% and 79% for pooled sera and FDs, respectively (see Table III), the number of sera or FDs to be collected and tested in pools to achieve an equivalent sensitivity to twelve single sera can be calculated as follows: 12/0.71 = 16.9 or 12/0.79 = 15.2 sera or FDs, respectively. For convenience, it was proposed to sample fifteen pigs per farm and to perform three tests on pools of either five sera or five FDs instead of twelve single tests on sera. Under these conditions, the sensitivity of the method on pooled sera and FDs compared to individual sera was found to be 89% and 98.7%, respectively. In breeding/finishing farms, ten sows and five finishing pigs were sampled (serum or FDs) instead of seven

Table IV
Results of an inter-laboratory comparison test to validate the use of pools of five filter discs for routine diagnosis of porcine respiratory and reproductive syndrome

ELISA	Sample identification	Type of sample	Regional veterinary diagnostic laboratories								Expected results*
			1	2	3	4	5	6	7	8	
Kit A	1	5 FD (5+)	+	+	+	+	+	+	+	+	+
	2	5 FD (4+/1–)	+	+	+	+	+	+	+	+	+
	3	5 FD (3+/2–)	+	+	+	+	+	+	+	+	+
	4 (=3)	5 FD (3+/2–)	+	+	+	+	+	+	+	+	+
	5 (=3)	5 FD (3+/2–)	+	+	+	+	+	+	+	–	+
	6	5 FD (2+/3–)	+	+	+	+	+	+	+	–	+
	7 (=6)	5 FD (2+/3–)	+	+	+	+	+	+	+	–	+
	8 (=6)	5 FD (2+/3–)	+	+	+	+	+	+	+	+	+
	9	5 FD (1+/4–)	+	–	+	+	+	–	–	–	–
	10	5 FD (5–)	–	–	–	–	–	–	–	–	–
	11	Serum	160	NT	160	320	160	NT	160	NT	160-320
	12	Eluted filter disc	16	16	16	NT	16	NT	16	16	8-32
Kit B	1	5 FD (5+)	+	+	+	+	+	+	+	+	+
	2	5 FD (4+/1–)	+	+	+	+	+	+	+	+	+
	3	5 FD (3+/2–)	+	+	+	+	+	+	+	+	+
	4 (=3)	5 FD (3+/2–)	+	+	+	+	+	+	+	+	+
	5 (=3)	5 FD (3+/2–)	+	+	+	+	+	+	+	+	+
	6	5 FD (2+/3–)	+	+	+	+	+	+	+	+	+
	7 (=6)	5 FD (2+/3–)	+	+	+	+	+	+	+	+	+
	8 (=6)	5 FD (2+/3–)	+	+	+	+	+	+	+	+	+
	9	5 FD (1+/4–)	+	+	+	+	+	+	+	+	+
	10	5 FD (5–)	–	–	–	–	–	–	–	–	–
	11	Serum	1,600	800	1,600	>3,200	800	800	800	800	800-1,600
	12	Eluted filter disc	40	40	40	10	20	80	40	40	20-80

*   expected results (result obtained in the laboratory of the authors)
FD: filter disc
NT: not tested
ELISA: enzyme-linked immunosorbent assay

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and five individual sera, respectively. The decision to sample more sows than finishing pigs was made because the seroprevalence of PRRS was generally higher in finishing pigs than in sows (data not shown).

Discussion

In this paper, the authors produce evidence that serological diagnosis of PRRS infection can be performed with high specificity and minimal loss of sensitivity by testing three pools of five sera or five FDs with commercial ELISA kits. The sensitivity of serum and FD pool detection compared to individual sera was estimated to be 71% and 79%, respectively. This lower sensitivity is a direct consequence of the dilution factor due to pooling. The difference in sensitivity between sera and FDs might be due to the nature of the samples. This assumption was confirmed by comparing detection on individual sera with that on the corresponding FDs. Thus, all FDs found to be positive were collected either on pigs scored positive for serum antibodies or on pigs scored negative but with a result close to the ELISA positive threshold (data not shown). Since all these pigs were from infected farms, the positive FDs can be reasonably assumed to have been true positive samples. This observation suggests that detection on FDs was actually improved compared to detection on sera.

The loss of sensitivity observed at the test level was balanced by an increase in the number of pigs tested. To ensure the same sensitivity as twelve single sera for detection of infection, the authors propose testing either three pools of five sera or five FDs. Under these conditions, the loss of sensitivity was found to be 11.2% and 1.3% with pools of sera and FDs, respectively. According to the authors, the lower sensitivity with pooled sera is probably not critical for PRRS diagnosis. Indeed, the samples used in this study were collected on farms that had been infected for more than six months (old infection). The number of positive pigs on these farms is expected to be lower than on recently infected farms or on farms where the virus is actively replicating and spreading. In other words, the detection sensitivity in serum pools should be sufficient to manage PRRS diagnosis in free areas or in areas where PRRS is still active and causing sanitary problems. In this study, the authors also showed that analysis on pooled samples was highly specific (>97.5%), thus enforcing the value of the method.

Filter discs can be easily collected from the ear or tail of pigs. This reduces sampling time and laboratory costs since three tests for fifteen FDs are performed instead of twelve individual tests. A comparative study of the costs of each diagnostic procedure demonstrated the advantage of the FD pools. Diagnosis involving pooled samples was two times cheaper than that of single sera. This diagnostic method was awaited by different regional sanitary organisations either to enforce their control strategy or to determine the prevalence of infection. The method is now used routinely for the serological monitoring of more than 2,000 farms in the 'Pays de la Loire' region where the prevalence of infection has, for three years, been maintained below 2% (6). Reduction of the cost of diagnosis can now be redirected to eradication processes on the remaining infected farms.

Filter discs were also successfully used for the diagnosis of Aujeszky's disease (AD) at the end of the 1980s and again in the 1990s (10). They reduced the costs of disease control in many regions that have now eradicated the infection. The possibility of using FDs is described in the instructions issued by the manufacturers, and in France, each ELISA kit for AD must be checked for sensitivity by a reference laboratory before being marked. For PRRS, there is no official requirement for the control of ELISA kits. However, to avoid variations in sensitivity between different ELISA batches, the authors have produced an FD standard, calibrated on the ELISA batches that were used to develop the method. This standard is delivered to regional laboratories for use in testing the sensitivity of new batches of ELISA. Filter discs have now been routinely used in France for 24 months for the detection of PRRS antibodies and so far, no difficulties have been encountered.

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Dépistage d'anticorps contre le virus du syndrome dysgénésique et respiratoire du porc sur disques en papier filtre

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Résumé

L'efficacité de deux trousses de dosage immuno-enzymatique (ELISA) a été évaluée pour le dépistage d'anticorps dirigés contre le virus du syndrome dysgénésique et respiratoire du porc dans des mélanges de sérums adsorbés sur des disques de papier filtre. Les procédures d'élution et d'incubation, de même que les seuils considérés comme positifs, ont été définis pour les deux trousses à l'aide d'échantillons recueillis sur des disques de papier filtre chez 60 porcs non infectés et 5 porcs présentant un titre d'anticorps peu élevé contre le virus. Le titre de 80 paires d'échantillons (sérum/disque de papier filtre) prélevés sur des porcs infectés a été déterminé avec les deux trousses de dosage immuno-enzymatique. Les auteurs ont ainsi démontré qu'il était possible de regrouper cinq sérums ou cinq disques de papier filtre lors d'une même analyse sans perte significative de sensibilité. Par rapport au dosage d'échantillons sériques individuels, la méthode présentait une sensibilité et une spécificité de 79 % et 97,5 %, respectivement. Ces valeurs ont été calculées sur la base de 200 mélanges de disques de papier filtre issus de 15 élevages infectés par le syndrome dysgénésique et respiratoire du porc et de 120 mélanges provenant de 71 élevages non infectés. On pourra compenser la perte de sensibilité en analysant deux mélanges de cinq échantillons prélevés sur des truies et un mélange de cinq échantillons provenant de porcs en période de finition, comme alternative au dosage respectif de 5 et de 7 sérums individuels.

Mots-clés

Diagnostic – Dosage immuno-enzymatique – Disque de papier filtre – Ensemble – Porc – Syndrome dysgénésique et respiratoire du porc.

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Detección en discos de papel filtro de anticuerpos contra el síndrome disgenésico y respiratorio porcino

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Resumen

Los autores evaluaron el rendimiento de sendos kits de ensayo inmunoenzimático (ELISA) para detectar anticuerpos contra el síndrome disgenésico y respiratorio porcino (SDRP) en mezclas de muestras séricas y de discos de papel filtro. Utilizando discos con muestras de sesenta cerdos no infectados y de cinco cerdos con títulos bajos de anticuerpos, se determinaron los procedimientos de elución e incubación y el umbral de positividad de ambos ensayos. Se titularon con los dos ELISA ochenta muestras apareadas (suero-disco) de cerdos infectados. Los autores demostraron así que cabía reunir cinco sueros o discos en un solo ensayo sin pérdida significativa de sensibilidad. A partir de los datos de 200 mezclas de discos con muestras procedentes de 15 granjas infectadas y de otras 120 con muestras de 71 granjas no infectadas, y en comparación con el

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análisis de sueros individuales, la sensibilidad de ese método resultó de un 79% y su especificidad de un 97,5%. Para compensar la pérdida de sensibilidad pueden someterse a prueba dos mezclas de cinco muestras de cerdas y una mezcla de cinco muestras de cerdos de engorde, como alternativa al análisis por separado de siete y cinco sueros individuales, respectivamente.

Palabras clave

Cerdo – Diagnóstico – Disco de papel filtro – Ensayo inmunoenzimático – Mezcla de muestras – Síndrome disgenésico y respiratorio porcino.

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