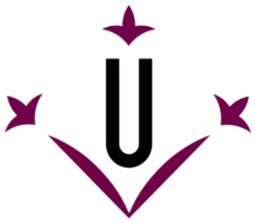

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Short Communication

Comparison of the immunoperoxidase monolayer assay and three commercial ELISA tests for detection of antibodies against porcine circovirus type 2

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Abstract

The aim of this study was to compare and correlate antibody titres against porcine circovirus type 2 (PCV2) in porcine sera (n = 1270) obtained by immunoperoxidase monolayer assay (IPMA) with the results of three commercial ELISAs (designated E1, E2 and E3). The correlation between IPMA and ELISA results was excellent ($r^2 \geq 0.90$). Compared to IPMA, E2 had the highest sensitivity (93.0%), followed by E3 (90.1%) and E1 (85.0%); the specificity was 100% for all tests. All three commercial ELISAs had predictive values similar to those of IPMA and could be used to monitor antibody responses against PCV2 infection and/or vaccination.

Keywords: Porcine circovirus type 2; Serology; Immunoperoxidase monolayer assay; ELISA

The immunoperoxidase monolayer assay (IPMA) is used widely for detection of antibodies against porcine circovirus type 2 (PCV2) (Opriessing et al., 2007; Fort et al., 2009; Fraile et al., 2012a and b). Neutralising antibodies (NAs) are the main antibodies responsible for protection and clearance of PCV2 infection (Meerts et al., 2005; Fort et al., 2007). Since there is a positive correlation between IPMA titres and titres of NAs (Fort et al., 2008, 2009), IPMA titres might be considered to be an indirect measure of NAs. Furthermore, high levels of maternally derived antibodies (IPMA titres ≥ 10 $\log_2$) appear to interfere with the development of humoral immunity after vaccination, while IPMA levels < 8 $\log_2$ do not have this effect (Fort et al., 2009; Fraile et al., 2012a, 2012b).

IPMA is relatively complex technique for routine diagnostic use, since it depends on the availability of virus infected cell cultures, requires a high level of technical expertise and is relatively slow for screening large numbers of sera. For these reasons, replacement of IPMA by automated serological tests with an objective end-point reading system, such as ELISAs, is desirable. The aim of this study was to compare and correlate the antibody titres determined by IPMA with the results of three commercial ELISAs and to determine if ELISA results can be useful to infer IPMA titres.

Sera used in this study (n = 1248) came from a previous study performed on a commercial farm with a previous diagnosis of PCV2 systemic disease (formerly known as postweaning multisystemic wasting syndrome) (Fraile et al., 2012b). One week before mating, 57 sows were randomly divided into two groups: (1) vaccinated sows (V; n = 26) receiving an intramuscular dose of Porcilis PCV (Intervet International BV) and unvaccinated sows (not vaccinated, NV, n = 31) receiving phosphate buffered saline (PBS) as placebo. At 4 weeks of age, 208 healthy piglets from these sows were divided into two groups; 106 piglets were vaccinated with Porcilis PCV and 102 piglets received only PBS. The following groups were included in the analysis: (1) NV piglets from

NV sows (NV-NV, *n* = 50); (2) V piglets from NV sows (NV-V, *n* = 52); (3) NV piglets from V sows (V-NV, *n* = 52); and (4) V piglets from V sows (V-V, *n* = 54). Blood samples were collected from piglets at 4, 12, 16, 21 and 26 weeks of age. In addition, 22 sera from 7- to 10-day-old Caesarean-derived, colostrum-deprived (CDCD) piglets were included in the study as negative controls. Animal care and study procedures were conducted in accordance with the guidelines of Good Experimental Practice, under the approval of the Ethical and Animal Welfare Committee of the Universitat Autònoma of Barcelona (Reference 665M2; approval on October 24th, 2013).

To detect anti-PCV2 antibodies, all serum samples (*n* = 1270) were analysed by IPMA and three commercial ELISAs: (1) SerELISA PCV2 Ab Mono Blocking (Synbiotics; E1); (2) Ingezim Circo IgG 11. PCV.K1 (Ingenasa; E2); and (3) PCV2 ELISA SK105 (Biochek; E3). Serum samples were analysed by IPMA in four-fold dilutions from 1:20 to 1:20,480 (Rodríguez-Arrioja et al., 2000). Results were expressed as $\log_2$ of the inverse of titre values. All samples with ≥ 4.32 $\log_2$ titre were considered to be positive. ELISAs were performed following the manufacturers' instructions and sera were analysed at 1:1000, 1:200 and 1:50 dilutions by E1, E2 and E3, respectively.

ELISA values were expressed as antibody titres according to the mathematical formula provided by each manufacturer and the corresponding plate-specific cut-off values were applied to convert the ELISA results into categorical data. Results for E1 (blocking ELISA) were converted to reciprocal optical density (OD) values (RecOD, calculated by subtracting the inverse of the OD of negative control samples to the inverse of the OD of the test sample). Results for E2 and E3 (indirect ELISAs) were expressed as S/P values (S = sample optical density, OD; P = mean positive control OD).

Average ELISA RecOD, S/P values and titres corresponding to each IPMA result, as well as Pearson (r) and determination (R^2) coefficients between serological values, were calculated for the three ELISA tests. The equation line ($y = ax + b$) and curve ($y = ax^2 + bx + c$) that best represented the relationships between IPMA and ELISA values was adjusted to allow the IPMA titre to be inferred from any ELISA result.

To ascertain if commercial ELISAs are reliable potential substitutes of IPMA, the sensitivity (Se) and specificity (Sp) of each ELISA kit was calculated using IPMA qualitative results as indicators of the true positive status of samples. The overall percentage of agreement (number of positive plus negative coincident results divided by the number of estimations), along with the Kappa coefficients (κ values), among serological assays were calculated for paired tests. The average values of antibody titres, RecOD and S/P ratios for each treatment group at different sampling times were used to generate antibody profiles. Antibody profiles of different groups for each serological technique were compared with the Mann-Whitney U test.

Table 1 summarises IPMA results and corresponding average values for ELISA RecOD, S/P values and titres. As indicated by the R^2 value, the correlation between IPMA and E1 results was > 0.92 for RecOD and ELISA values when both linear and polynomial equations were considered (Fig. 1). Considering a linear relationship between IPMA and E2 results, the correlation was low for ELISA titres ($R^2 = 0.48$) and relatively high for S/P ratio values ($R^2 = 0.7$) (Fig. 1). Nevertheless, R^2 increased to 0.91 and 0.97 for E2 titres and S/P ratios, respectively, when a polynomial equation was considered (Fig. 1). The coefficient of correlation was > 0.88 for both S/P and E3 titres when a linear equation was considered (Fig. 1). R^2 increased to 0.90 for both S/P values and E3 titres when the polynomial equation was used (Fig. 1).

All serum samples were PCV2 IPMA positive; CDCD pig sera resulted negative. E2 had the highest Se (93.02%), followed by E3 (90.14%) and E1 (85.02%). Serum samples from CDCD pigs were negative in all three ELISAs; thus Sp was 100% for all ELISAs. The overall percentage of agreement and κ coefficients among ELISAs are shown in Table 2. More significant differences in antibody profiles among treatment groups were evident using E2 than IPMA, E1 or E3 (Fig. 2).

All ELISAs had good diagnostic accuracy and could be used to discriminate and interprete the antibody profiles obtained at farm level, although the agreement between ELISAs was fair to moderate. On the basis of the determination coefficient, the correlations between IPMA and the three ELISAs were excellent ($R^2 \geq 0.90$). It was possible to infer an IPMA titre from each RecOD or S/P value, and vice versa, by means of linear or polynomial equations. The equivalence between IPMA and ELISA results should be useful for monitoring antibody titres due to PCV2 infection and vaccination. According to observations by Fort et al. (2009) and Fraile et al. (2012a and 2012b), maternally derived IPMA titres $\geq$ 10 $\log_2$ interfere with seroconversion following PCV2 vaccination. The present study shows that ELISA values can be used to infer IPMA titres in order to select the age at vaccination that prevents this interference.

All tests used in the present study (IPMA, E1, E2 and E3) were able to detect the presence of maternally derived antibodies at 4 weeks of age based on antibody profiles. At this sampling time, piglet groups from vaccinated sows had the highest calculated antibody titres in all ELISAs and by IPMA. Passive antibodies waned and reached their minimum level between 8 and 12 weeks of age, depending on the treatment received by the sow and the antibody values at weaning. The three ELISA tests, as well as the IPMA technique, also detected seroconversion by 21 weeks of age. In contrast to the other serological assays used in this study, E3 was not able to detect overt seroconversion due to piglet vaccination at 8 weeks of age.

E2 was the most suitable kit to detect significant differences between treatment groups, followed by IPMA, E1 and E3. The differential ability to detect seroconversion due to piglet vaccination and to discriminate between vaccine treatment groups could be a consequence of the different types of antibodies detected by each assay. The types of antibodies detected by E1 and E3 are not clear. Considering the intrinsic characteristics of a blocking ELISA, we speculate that total antibodies were detected by E1, whereas E2 only detects IgG, as indicated by its manufacturer. In the case of IPMA, a peroxidase-conjugated protein A was used to develop the reaction, also specifically detecting porcine IgG antibodies. This should be kept in mind when comparing and interpreting test results within and between laboratories (Patterson et al., 2011).

In conclusion, the results of the present study indicate that all three commercial ELISA assays provided predictive values similar to those offered by IPMA and represent reliable potential substitutes for IPMA to monitor antibody responses against PCV2 infection and/or vaccination in quantitative and qualitative terms.

Conflict of interest statement

ELISA kits evaluated in this study were donated by Synbiotics, Ingenasa and Biochek. The study design, analysis of results and writing of the manuscript was solely performed by the authors of the paper. None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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Table 1

Average RecOD, S/P ratio values and calculated ELISA titres for each immunoperoxidase monolayer assay (IPMA) titre and the corresponding 95% confidence intervals.

IPMA titre	Number of samples	E1		E2		E3	
		RecOD	Titre	S/P	Titre	S/P	Titre
4.32	9	0.15 [-0.06;0.35]	318 [138;498]	0.98 [0.48;1.48]	12117 [-3887;28122]	0.97 [0.50;1.44]	2279 [1101;3457]
6.32	73	0.16 [0.10;0.22]	432 [334;529]	0.59 [0.51;0.68]	1440 [83;2795]	0.85 [0.73;0.97]	1972 [1679;2266]
8.32	213	0.35 [0.28;0.41]	618 [537;700]	0.83 [0.77;0.89]	3552 [1835;5269]	1.03 [0.95;1.11]	2420 [2214;2625]
10.32	310	0.66 [0.60;0.72]	987 [903;1072]	1.11 [1.06;1.16]	4869 [3919;5819]	1.35 [1.28;1.41]	3223 [3051;3396]
12.32	297	0.89 [0.83;0.96]	1322 [1226;1418]	1.38 [1.33;1.43]	16610 [10364;22857]	1.53 [1.46;1.59]	3692 [3524;3859]
14.32	345	0.87 [0.81;0.92]	1545 [1453;1636]	1.68 [1.63;1.74]	45004 [27385;62623]	1.63 [1.55;1.67]	3908 [3752;4064]

Lower and upper limits of the confidence intervals are shown between brackets.

Table 2

Overall percentage of agreement and κ coefficients between ELISAs.

	E2						E3					
E1	Positive	Negative	Total	Agreement	κ*	Kmax**	Positive	Negative	Total	Agreement	κ*	Kmax**
Positive	1024	37	1061				995	66	1061			
Negative	137	72	209				130	79	209			
Total	1161	109	1270	86.3%	0.383 [0.311;0.455]	0.731	1125	145	1270	84.6%	0.360 [0.289;0.430]	0.698
E2												
Positive							1086	75	1161			
Negative							39	70	109			
Total							1125	145	1270	91.0%	0.502 [0.423;0.581]	0.821

*Lower and upper limits of the confidence intervals of κ coefficient are shown between brackets.

**The maximum obtainable Kappa (Kmax) reflects how much an imbalanced positive and negative classification constrains the agreement between techniques, being the prevalence of the classifications one of the factors that could reduce the K value.

212 **Figure legends**

213

214 Fig. 1. (A) Correlation (R^2) between average immunoperoxidase monolayer assay (IPMA) titres and
215 average ELISA RecOD or S/P values of commercial porcine circovirus type 2 ELISA kits E1, E2
216 and E3, respectively. (B) Correlation (R^2) between average IPMA and ELISA titres. Linear ($y = ax + b$) and polynomial ($y = ax^2 + bx + c$) equations defined the lines (continuous) and curves (dashed),
218 respectively, that best represented the relationship between IPMA and ELISA values for each
219 serological technique.

220

221 Fig. 2. Antibody profiles of the different treatment groups obtained by immunoperoxidase
222 monolayer assay (IPMA) and ELISAs E1, E2 and E3 (panels A, B, C and D, respectively). Mean
223 and standard error (indicated by error bars) of serological values for each group and sampling time
224 is represented in each figure.

Figure 1

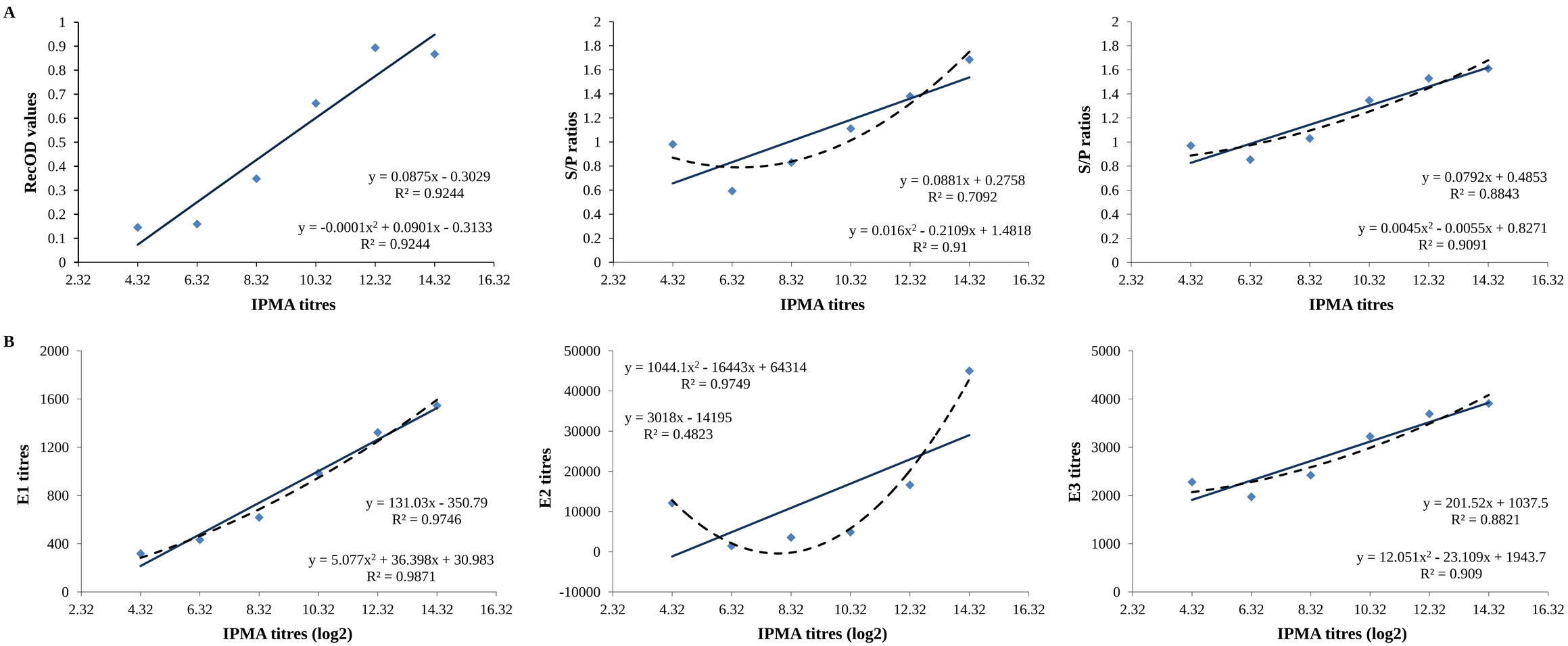
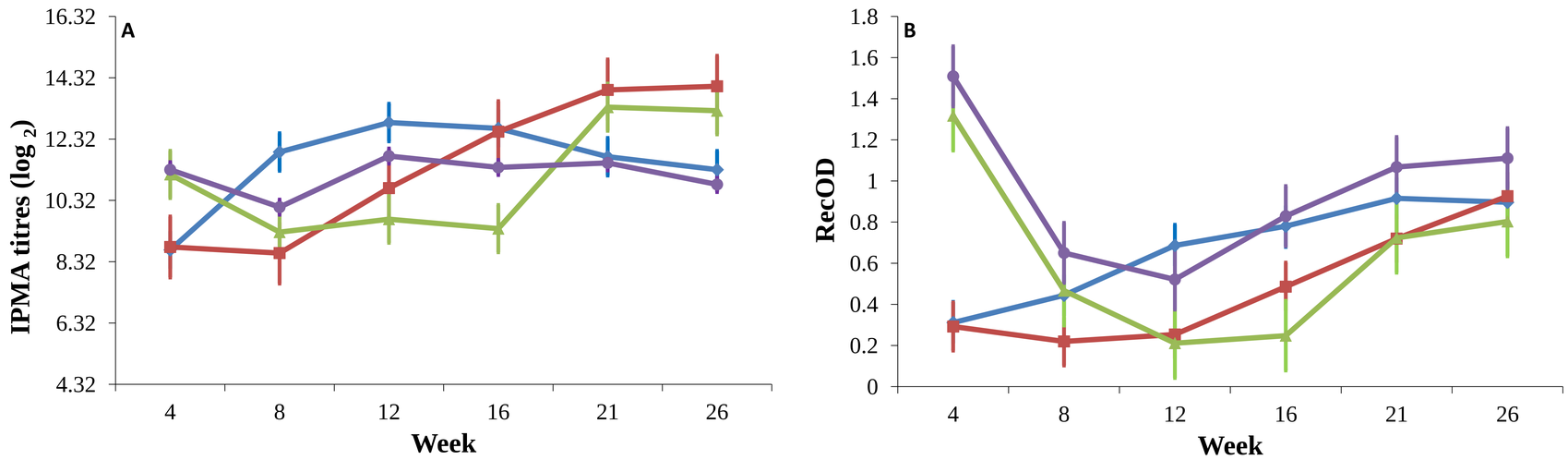

A
RecOD values
y = 0.0875x - 0.3029
R² = 0.9244
y = -0.0001x² + 0.0901x - 0.3133
R² = 0.9244
IPMA titres
S/P ratios
y = 0.0881x + 0.2758
R² = 0.7092
y = 0.016x² - 0.2109x + 1.4818
R² = 0.91
IPMA titres
S/P ratios
y = 0.0792x + 0.4853
R² = 0.8843
y = 0.0045x² - 0.0055x + 0.8271
R² = 0.9091
IPMA titres
B
E1 titres
y = 131.03x - 350.79
R² = 0.9746
y = 5.077x² + 36.398x + 30.983
R² = 0.9871
IPMA titres (log2)
E2 titres
y = 1044.1x² - 16443x + 64314
R² = 0.9749
y = 3018x - 14195
R² = 0.4823
IPMA titres (log2)
E3 titres
y = 201.52x + 1037.5
R² = 0.8821
y = 12.051x² - 23.109x + 1943.7
R² = 0.909
IPMA titres (log2)

Figure 2


A
IPMA titres (log 2)
16.32
14.32
12.32
10.32
8.32
6.32
4.32
4
8
12
16
21
26
Week
B
RecOD
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
4
8
12
16
21
26
Week
C
S/P ratio
2.4
2.1
1.8
1.5
1.2
0.9
0.6
0.3
0
4
8
12
16
21
26
Week
D
S/P ratio
2.4
2.1
1.8
1.5
1.2
0.9
0.6
0.3
0
4
8
12
16
21
26
Week
NV-NV
NV-V
V-NV
V-V