Assessment of three commercial ELISAs for the detection of antibodies against *Porcine epidemic diarrhea virus* at different stages of the immune response.

Díaz I [1,2*], Pujols J [1,2], Cano E [1,2], Cuadrado R [1,2], Navarro N [1,2], Mateu E [2,3,4,], Martín M [2,3,4]

[1] IRTA, Centre de Recerca en Sanitat Animal (CReSA, IRTA-UAB), Campus de la Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain.

[2] OIE Collaborating Centre for the Research and Control of Emerging and Re-Emerging Swine Diseases in Europe (IRTA-CReSA), 08193 Bellaterra, Spain.

[3] Departament de Sanitat i Anatomia Animals, Universitat Autònoma de Barcelona (UAB), 08193 Bellaterra, Spain.

[4] Centre de Recerca en Sanitat Animal (CReSA, IRTA-UAB), UAB, Campus de la Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain.

* Corresponding author. Ivan.diaz@irta.cat

Highlights

- Commercial PEDV ELISAs showed significant differences in sensitivity.
- Five months after PEDV inoculation, the rate of detection ranged from 0% to 92%.
- The competitive ELISA was the only one that detected antibodies in a high percentage of animals for up to five months.
- Differences between ELISAs could be related to the antigens and to the cut-offs.

Abstract

Three commercial ELISAs -two based on spike (E1 and E3) and one on nucleocapsid protein (E2)- were used to analyze the development and persistence of antibodies against *Porcine epidemic diarrhea virus* (PEDV). Seventy-five four-week-old PEDV-negative piglets were inoculated orally with a European G1b PEDV (INOC) and fourteen were kept as controls (CTRL). After the inoculation, E3 detected positive animals as soon as 7 days post inoculation (dpi), while the earliest detection with E1 and E2 was at 14 dpi. All samples were positive at 21 and 28 dpi using E1 and E3, respectively, while E2 failed to detect 23.3% of the inoculated pigs at any time point. The percentages of positive samples were different through the study: E1 and E3 > E2 from 14 to 56 dpi; and E3 > E1 > E2 from 56 to 154 dpi ($P<0.05$). Five months after the inoculation, E3 still detected 92.0% ($IC_{95\%}$=85.1-98.8%) of pigs as positive, while E1 and E2 detected only 27.0% ($IC_{95\%}$=16.0-37.9%) and 0%, respectively. The sensitivity for E2 never exceeded 0.62. Specificity was 1 for all ELISAs. These different outcomes could be related to the ELISA strategies (indirect versus competition), the antigens used, the cut-off, or to other intrinsic factors of each test. The observed differences could be of importance when assessing whether older animals, such as fatteners or gilts, had previously been in contact with PEDV.

Keywords: ELISA, *Porcine epidemic diarrhea virus* (PEDV), Spike, Nucleocapsid

1. Introduction

Porcine epidemic diarrhea virus (PEDV) is an enveloped, positive-sense, single-stranded RNA virus belonging to the genus *Alphacoronavirus*, family *Coronaviridae*, order *Nidovirales* (ICTV, 2011). In pigs causes a highly transmissible enteric disease characterized by acute watery diarrhea that is especially severe in suckling piglets -case-fatality rate may reach 100% (Geiger and Connor, 2013; Jung and Saif, 2015)-. PEDV was firstly described in UK and Belgium in the 1970s (Pensaert and de Bouck, 1978), causing diarrhea outbreaks with high mortality in neonates. Afterwards, PEDV spread in many countries where it established endemically, with sporadic outbreaks of varying severity (Carvajal et al. 2015, Martelli et al., 2008).

Based on their S gene sequences, PEDV strains can be differentiated into G1a, G1b, G2a and G2b (Chen et al., 2012; Lee, 2015). Most of the PEDV originally present in Europe in the 70s are classified as G1a, whilst the most recent ones -from 2010 onwards -, which can also be found in Asia and North America, are G1b (Hanke et al., 2017; Kocherhans, et al., 2001; Theuns et al., 2015). In 2013, a new introduction of PEDV in North America caused the death of millions of piglets, being particularly devastating for the US and Canadian pig industries (Schulz and Tonsor, 2015; Weng et al., 2016). The strain causing those outbreaks in America could be traced back to Asia (Huang et al., 2013). Thus, differences in pathogenicity of recent genogroups have been demonstrated; G1b strains (known as S-INDEL) present low to moderate virulence, while G2b (non S-INDEL), isolated only in Asia and North-America, show a higher virulence (Carvajal et al. 2015).

Diagnosis of PEDV outbreaks is mostly done by RT-PCR using feces (de Arriba et al., 2002). On the contrary, the measurement of antibodies against PEDV by ELISA can be useful to detect positive animals beyond the excretion period for different purposes, such as to know the possible presence, persistence and spread of the virus within the farm, the screening of the status of an animal source, to check the immune status of gilts and sows before farrowing, as well as for immunological and epidemiological studies on PEDV.

ELISAs currently commercialized for the detection of antibodies against PEDV are based on the spike or the nucleocapsid proteins. The aim of the present study was to compare the performance of three PEDV commercial ELISAs -two based on spike protein (E1 and E3) and one on nucleocapsid protein (E2)- using sera from animals experimentally infected with a G1b PEDV strain in a long-term study.

2. Material and methods

2.1 Experimental design

Animals and samples used in this study were part of a larger project aimed to characterize the duration of the immune response after the challenge with an European G1b PEDV S-INDEL strain. Briefly, the present study used 89 three-week old piglets from a PEDV-negative source determined by quantitative PCR (qPCR; VetMAX Swine Enteric Panel TGEV/PEDV/PRV-A kit; Thermo Fisher Scientific Inc., Madrid, Spain) and ELISA (INgezim PEDV; 11.PED.K1; Eurofins INGENASA, Madrid, Spain). Animals were transported to experimental facilities, ear-tagged and randomly distributed into two separated groups: inoculated (INOC, n=75) and controls (CTRL, n=14). All animals were intramuscularly injected with Ceftiofur (3 mg/kg; EXCENEL, Zoetis, Hostalnou de Bianya, Spain) to prevent diarrhea by *E.coli*. After one week of acclimation, namely at 0 days post-inoculation (dpi), piglets in INOC group were orally inoculated using a gastric cannula with 2 mL of the G1b PEDV Calaf-1 (2014) (GenBank accession number MT602520), at a dose of cycle threshold (Ct) =14.7, which corresponded to $10^{4,3}$ tissue culture infective dose per mL ($TCID_{50}$/mL). Animals in CTRL group were kept mock-infected with PBS.

All experiments involving pigs were done under the approval of Ethical Committee of IRTA and authorized by the Catalan Government (Ref. CEO-H/9450). Animals were kept in approved experimental facilities and were subjected to veterinary supervision for health and welfare. Pig handling was done by veterinarians and trained personnel that fulfilled the Spanish and European Union requirements.

2.2 Clinical follow-up and sampling

The appearance of individual feces was scored in a scale ranging from 0 (normal) to 3 (severe diarrhea). Hence, the maximum value for sum of scores at a given day was 225 (75 animals x 3). Individual serum samples were collected before virus inoculation and weekly until day 42 and then at 56, 78, 105, 133 and 154 dpi. Individual fecal samples were also collected at the same time points to assess the presence of PEDV.

2.3 PEDV genome detection in feces

The presence of PEDV in feces was determined by qPCR (VetMAX Swine Enteric Panel TGEV/PEDV/PRV-A kit, Thermo Fisher Scientific Inc.), using the Path-ID Multiplex One-Step RT-PCR kit (Thermo Fisher Scientific Inc.). Viral RNA was extracted using MagMAX pathogen RNA/DNA (Thermo Fisher Scientific Inc.) and a BioSprint 96 workstation (Qiagen Iberia, Las Matas, Spain), according to the manufacturer's instructions. Positive and negative controls were included in each reaction and round of RNA extraction. Results were expressed as Ct.

2.4 ELISAs

Three commercial ELISAs for detection of anti-PEDV antibodies were considered: E1 (INgezim PEDV; 11.PED.K1; Eurofins INGENASA), E2 (ID Screen PEDV indirect; IDVet, Grabels, France), and E3 (ID Screen PEDV Spike Competition; IDVet, Grabels, France). Both E1 and E2 were indirect ELISAs while E3 was a competition ELISA. E1 and E3 used as antigen spike glycoprotein from G1b strains, while E2 was based on a G1b nucleocapsid protein.

All tests were performed and interpreted according to the manufacturer's instructions. For E1, results were expressed as a sample-to-positive (S/P) ratio, namely a ratio of the optical density (OD) of a given sample over the OD of the positive control provided with the kit. An S/P ratio ≥ 0.35 was considered positive. For E2, S/P percentages (S/P%) were calculated: [(DO sample – DO negative control) / (DO positive control – DO negative control)] x 100. In this case, the threshold to consider a sample positive was > 60%. Since E3 was a competition ELISA, results were expressed as a sample-to-negative (S/N) percentage [(DO sample/DO negative control) x 100]; results lower than 50% were considered positive.

For any given ELISA, all samples yielding doubtful results were retested to discard any potential error attributable to the laboratory processing. In order to minimize biases, all samples were tested using the same ELISA batch.

2.5 Statistical analyses

Excel 2016 (Microsoft) was used to calculate descriptive statistics. Comparison of the percentages of positive pigs between ELISAs at a given sampling day was done using StatsDirect v3.2.8; firstly, a screening to assess if there were differences was done by Chi-square test for multiple groups and then, the pairs were tested by the Fisher's exact test.

3. Results and discussion

PEDV infection beyond the first days of life is often mild and/or of short duration (Crawford et al., 2015; Jung and Saif, 2015; Weng et al., 2016). In some cases, infection can remain unnoticed making an indirect detection, such as the measure of antibodies a useful screening tool. Several studies have evaluated both commercial and in-house ELISAs, either spike-based or nucleocapsid-based ones (Gerber et al., 2016; Okda et al., 2015; Strandbygaard et al., 2016). However, to our knowledge only one study has been published on the assessment of PEDV antibodies during a long period (Knuchel et al., 1992). In that case, two in-house ELISAs based on spike and nucleocapsid proteins were compared during six months after an experimental infection. According to the authors, antibodies against spike protein remained detectable for longer periods compared to the nucleocapsid ones. It is worth mentioning that the experimental infection was done with a G1a PEDV strain (V215/78) already present in

Europe in the 70s (Pensaert and de Bouck, 1978). The aim of the present study was to assess the dynamics of PEDV-specific production during five months, using three commercial ELISAs (two spike-based ELISAs and one nucleocapsid-based ELISA), after a European G1b PEDV strain experimental infection.

PEDV infection was demonstrated in all animals from INOC group, both by clinical observations and PEDV genome detection in feces. Regarding clinical scores, presence of loose stools/diarrhea was detected in all pigs. The maximum cumulative clinical score was 120 at day 4 dpi (out of a potential maximum of 225). No loose stools or diarrhea were observed beyond 21 dpi. Fecal excretion of PEDV was negative for all pigs at 0 dpi. All INOC piglets were positive at 7 dpi (mean Ct=24.3±4.0). Afterwards, the percentage of positive samples and the average amount of PEDV genome detected in positive animals declined until 42 dpi, when only one animal was still positive (Ct=36.7). Afterwards, all animals remained negative until the end of the study at 154 dpi. CTRL were all qPCR negative throughout the study.

Figure 1 summarizes the percentage of positive results detected by each ELISA. At the beginning of the study, all pigs both in INOC and CTRL groups were negative by all ELISAs. In the INOC group, 95.0% ($IC_{95\%}$=91.5-100%) of the animals were detected as positive at 14 dpi with E1, with all of them positive at 21 dpi. Using E2, the highest percentage of positive pigs was 62.2%, detected at 21 dpi ($IC_{95\%}$=51.1-73.2%). Importantly, with this E2, 19/75 INOC animals (23.3%; $IC_{95\%}$=16.3-36.9%) were never detected as positive (OD below the cut-off of the kit). When samples were analyzed using E3, 50.7% of the pigs ($IC_{95\%}$=39.4-62.0%) were classified as positive as soon as 7 dpi; at 28 dpi all were positive and remained in that way until 56 dpi. From 14 to 56 dpi, the percentages of positive samples were always significantly higher in E1 and E3 than in E2 ($P<0.05$). Afterwards, significant differences were observed between all ELISAs, with E3 being always the more sensitive (E3>E1>E2; $P<0.05$). Five months after the inoculation, E3 still detected as positive 92.0% ($IC_{95\%}$=85.1-98.8%) of the inoculated animals, while E1 and E2 detected only 27.0% ($IC_{95\%}$=16.0-37.9%) and 0%, positive animals, respectively.

Regarding S/P, S/P% or S/N% means (figures 2a-2c), the maximum value in INOC group was observed at 21 dpi for both E1 and E2, while the peak was seen at 28 dpi for E3. For E2, mean S/P% was slightly above the cut-off at 28 dpi and from that day until 154 dpi was always clearly below (figure 2b). The dynamics of the mean S/N% for E3 from 28 dpi onwards followed similar mean values (figure 2c).

The sensitivity (Sn) for each ELISA at a given sampling day was also calculated (figure 3). Sn was 1 for E1 at 21 dpi and for E3 at 28dpi; in contrast, for E2 Sn never exceeded 0.63. Later

on, Sn values declined for all ELISAs, although remained above 0.9 using E3. Mean S/N% values and Sn for E3 remained highly constant during all the experiment, especially when compared with E1 and E2. E3 was especially valuable for detecting individuals during longer periods, maintaining the capability to detect >90% of positive animals after five months. Thus, after a PEDV infection in the first days or weeks of life, E3 could still detect most animals as positive until the finishing phase. In comparison, E1 seemed to have some limitations for long-term detection and, therefore, it would be less useful to detect whether older animals, such as finishers or replacement gilts, had been infected at young ages. In contrast, specificity (Sp) was very high for all ELISAs (Sp=1).

The rationale behind the differences observed among the Sn of the three ELISAs are unclear. Regarding the antigens, E2 is based on the nucleocapsid, which is well-conserved among PEDV strains (Lin et al., 2015), is the most abundant viral protein and induces a strong humoral response (Li, 2015; Sturman and Holmes, 1983; Utiger et al., 1995). However, it has been suggested that the spike protein has a remarkably higher immunogenicity compared to nucleocapsid, and that spike-based ELISAs, such as E1 and E3, are more specific and sensitive than nucleocapsid-based ones (Hou et al., 2007; Knuchel et al., 1992; Okda et al., 2015). Morevorer, when spike and nucleocapsid-based ELISAs have been compared in the same PEDV-infected pigs, antibodies against spike have remained detectable in sera for longer periods (Knuchel et al., 1992). Interestingly, sera from PEDV-infected pigs not only could reach higher titers in spike-based ELISAs, but also these antibodies could closely link with neutralization activity and be, therefore, relevant for protection (Paudel et al., 2014).

The abovementioned facts may partially explain the presence of false negatives and the lowest Sn seen for E2 compared to E1 and E3. Conversely, some authors have claimed that differences among assays could be more related to intrinsic factors, rather than to the PEDV antigen used, as demonstrated using a combination of different antigens and different ELISA strategies (Gerber et al., 2016). Accordingly, differences observed between E1 and E3 (both spike-based ELISA) could be due to ELISA strategies (indirect versus competition) or to other intrinsic factors: cut-off values; coating with a recombinant versus purified spikes, or alternatively, to spike heterogeneity (Lin et al., 2015).

To determine if the low Sn calculated for E2 was due to the cut-off value (S/P% > 60%), a new one was calculated based on the results obtained from truly negative pigs in the present study [(n=243 observations: all samples in INOC group at day 0 dpi (n=75) plus all samples from 0 to 154 dpi in CTRL group (n= 14 pigs x 12 sampling days = 168)]. For this, mean S/P% + 5xSD

was calculated and set as a potential cut-off (99.9% of values for negative samples). In regards of Sn, figure 3 (E2', dotted gray line) shows the ability of E2' to detect positive animals using the new threshold (S/P% > 17%). The magnitude and pattern for the new Sn (E2') was like the one obtained for E1 and clearly higher than that calculated using the original threshold (E2). Using the new cut-off, all animals yielded positive results at 21 dpi, percentages of positives remained at 88.0% ($IC_{95\%}$=80.6-95.3%) at 56 dpi and 19.3% ($IC_{95\%}$=15.4-23.3%) at 154 dpi. Applying S/P% > 17% as cut-off, only one sample was detected as false positive (Sp=99.6%). It cannot be discarded that the original cut-off of E2 was higher because the field samples used during the standardization and validation process showed a different behavior in the ELISA, compared to experimental samples.

In summary, this study demonstrates the existence of significant differences between three commercial PEDV-specific ELISAs. As an example, E2 showed a very low Sn, with a 25% of false-negative animals throughout all the study. Differences were particularly marked during long-term phases after the infection. Thus, E3 still detected as positive more than 90% of the animals as latter as five months after the infection; while, at that time, E1 and E2 only detected 27% and 0%, respectively. The differences could be due to ELISA strategies (competition versus indirect), the antigen used and/or others intrinsic factors of each ELISA. This fact could be a challenge when monitoring the PEDV immune status in older animals, such as pigs during the final phase of fattening or gilts, if they were infected some months before.

Declaration of Competing Interest

The Authors declare no conflict of interest with respect to the research, authorship, and/or publication of this article.

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Figure 1. Percentage of positive results in each ELISA. Bars indicate the percentage of positive results in INOC group for E1 (black bar) for E2 (grey bar), and for E3 (white bar) at a given sampling day. Bars with a letter above show significant differences among ELISAs at a given sampling day (Fisher's exact test; <0.05).

Figure 2. Longitudinal serological profiles. Lines indicate the mean ± SD results at a given sampling day. In figure 2a, means are shown as mean of the S/P ratios (E1); in figure 2b, means of the S/P% (E2); and in 2c, means of the S/N% (E3). Black lines correspond to INOC group and dashed grey lines to CTRL group. The dotted black lines show the threshold value for each ELISA.

Figure 3. Sensitivity (Sn) for each ELISA throughout the study. Lines indicate the Sn values of each ELISA at different days: black line (E1), dashed grey line (E2) and grey line (E3). Dotted grey line (E2') indicates the Sn for E2 using a recalculated cut-off.

Figure 1

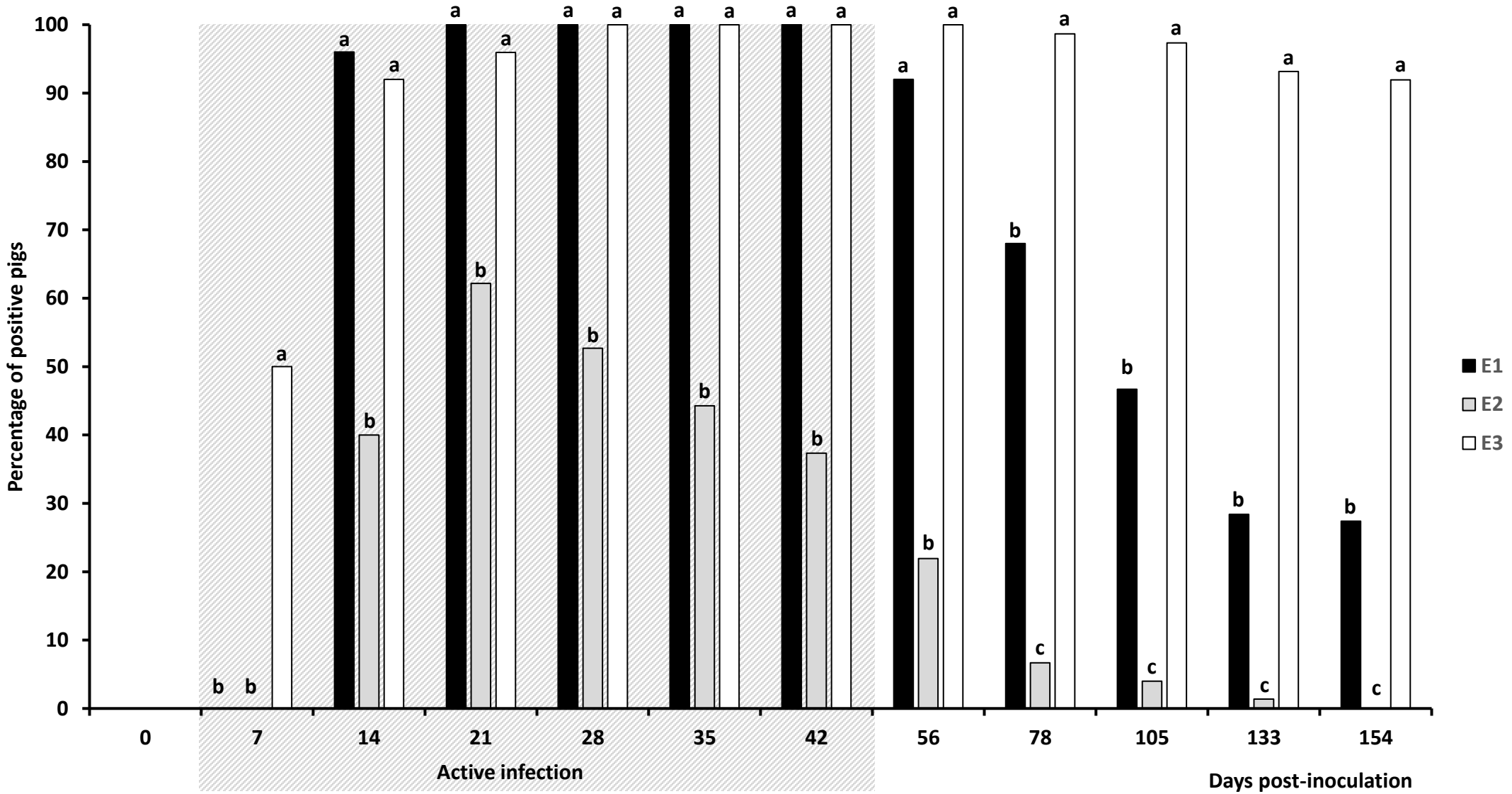

Percentage of positive pigs
100
90
80
70
60
50
40
30
20
10
0
0
7
14
21
28
35
42
56
78
105
133
154
Active infection
Days post-inoculation
E1
E2
E3

Figure 2a

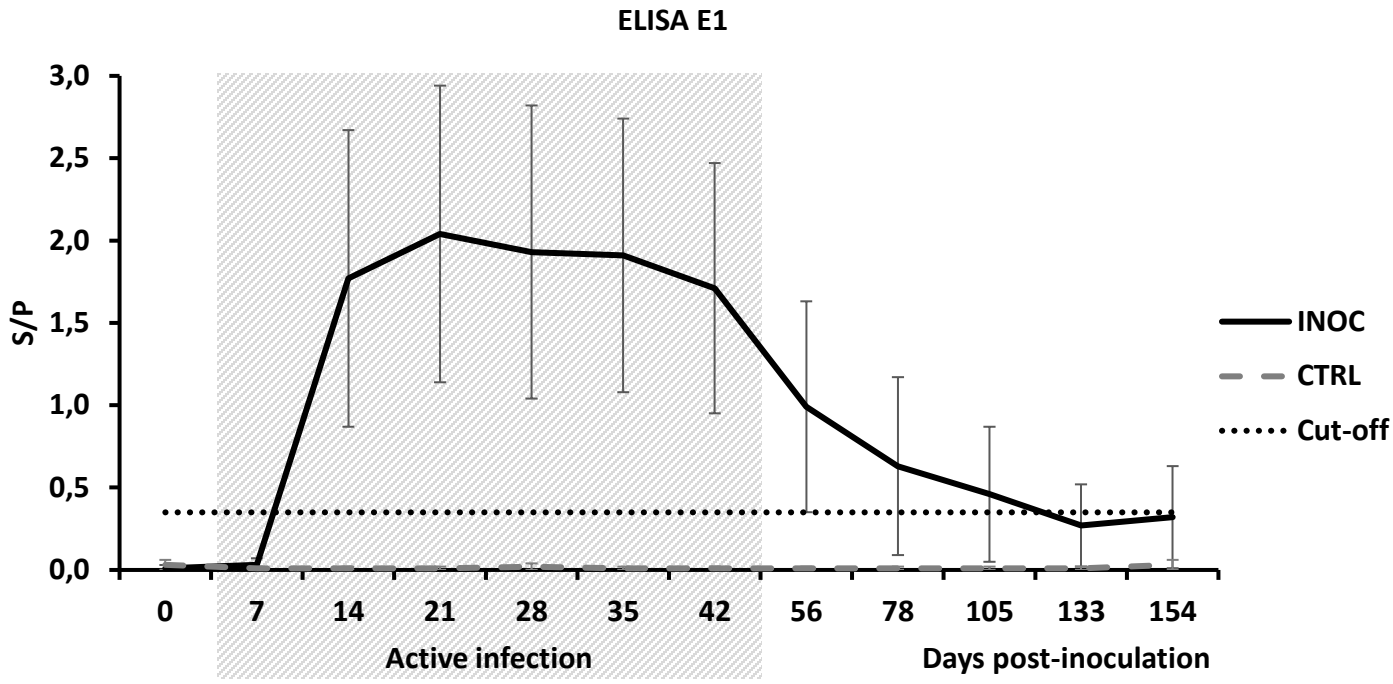


Figure 2b

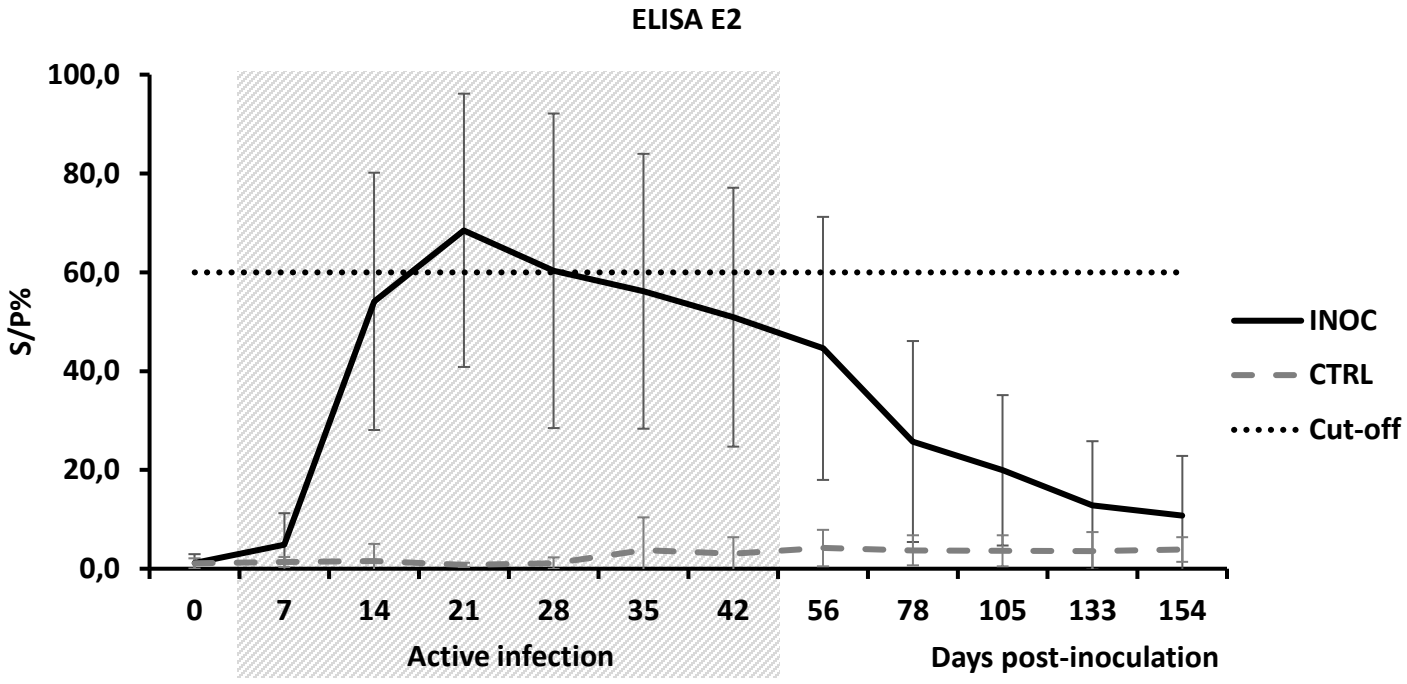


Figure 2c

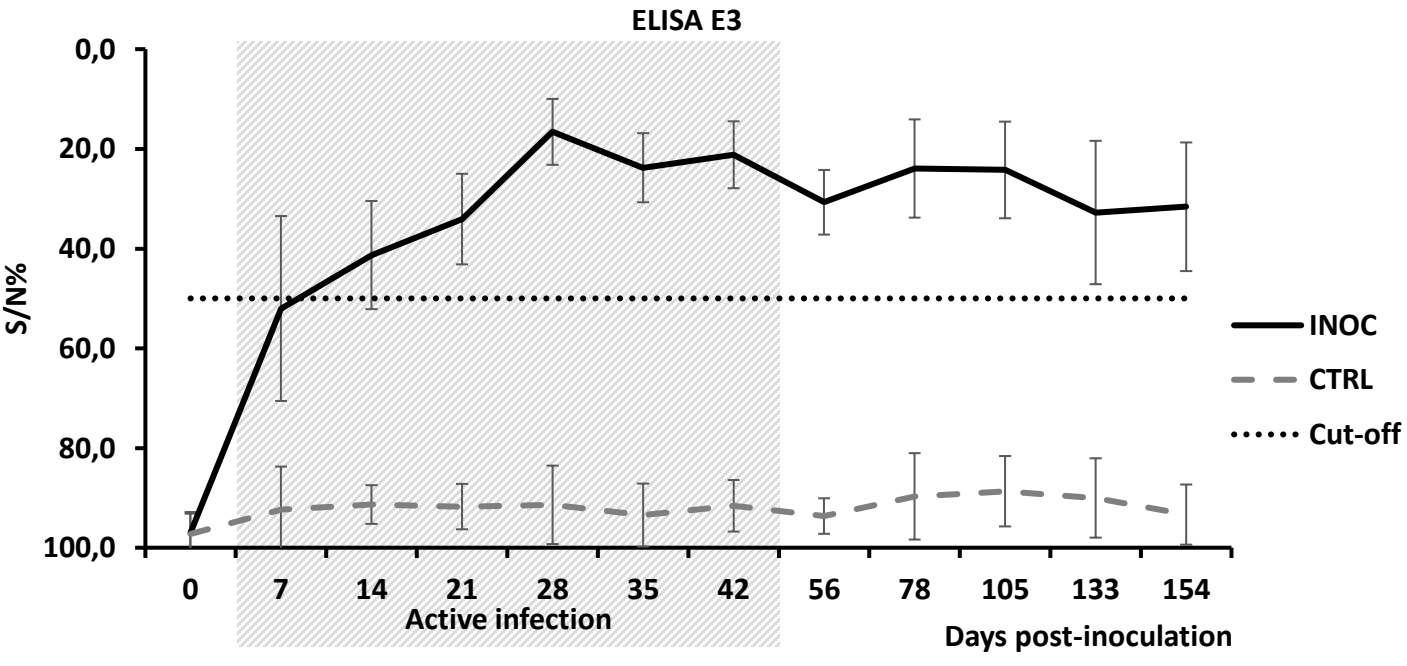
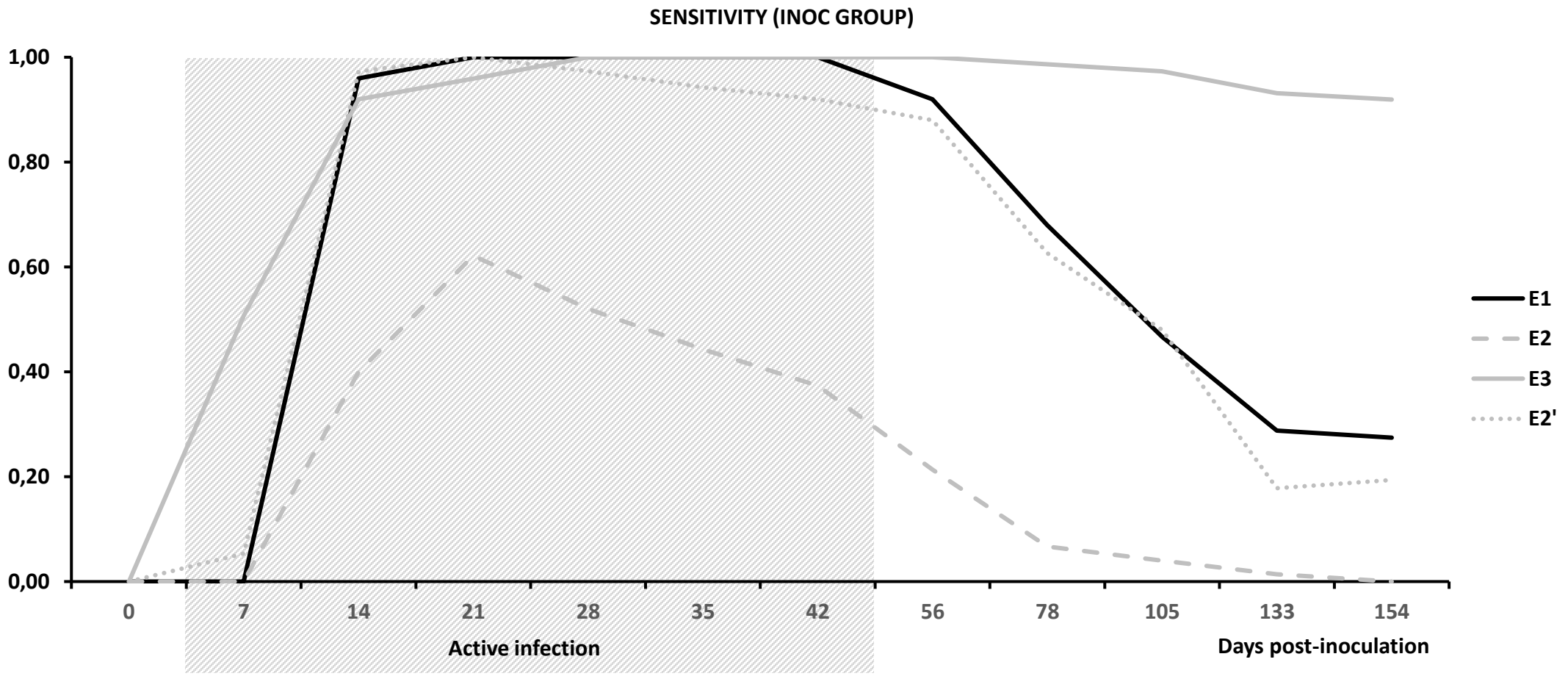


Figure 3


SENSITIVITY (INOC GROUP)
1,00
0,80
0,60
0,40
0,20
0,00
0
7
14
21
28
35
42
56
78
105
133
154
Active infection
Days post-inoculation
E1
E2
E3
E2'