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## Technical Note

# Agrodiag PorCoV: A multiplex immunoassay for the differential diagnosis of porcine enteric coronaviruses



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## ARTICLE INFO

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## ABSTRACT

Three different porcine enteric coronaviruses (PECs), i.e., porcine epidemic diarrhea virus (PEDV), transmissible gastroenteritis virus (TGEV) and porcine Deltacoronavirus (PDCoV) are currently circulating in U.S. commercial swine herds. Differential diagnosis of PECs relies on laboratory methods. This study describes the development of an ELISA-like multiplex planar immunoassay based on virus-specific recombinant S1 proteins printed in an array of spots at the bottom of a 96-well microplate for simultaneous detection differential serodiagnosis of PEDV, TGEV, PDCoV in a single sample. The technology overall format and working principle is similar to the solid-phase standard ELISA. After the three typical incubation steps, the reaction was visualized as blue spots which intensity correlated with antibody levels to specific viral antigen target in the array. The diagnostic performance of the assay was evaluated on known status serum samples ( $n = 480$ ) collected over time (day post-inoculation -7, 0, 7, 14, 21, 28, 35, and 42) from pigs inoculated with PEDV, TGEV Purdue, TGEV Miller, PDCoV (USA/IL/2014), or mock inoculated with culture media under experimental conditions. Antigen-specific cut-offs were selected to ensure 100% diagnostic and analytical specificity for each given antigen target. The overall diagnostic sensitivity was 92% (44/48 positives, 95% confidence interval (CI) 98,100) for PEDV S1, 100% (95/95 positives, 95% CI 98, 100) for TGEV S1, and 98% (47/48 positives, 95% CI 97, 100) for PDCoV S1. The results of this study demonstrate that the AgroDiag PEC multiplex immunoassay is an efficient and reliable test for differential detection and serodiagnosis of PEDV, TGEV and PDCoV.

## 1. Introduction

Three different porcine enteric coronaviruses (PECs), order *Nidovirales*, family *Coronaviridae*, are currently circulating in commercial swine herds, including transmissible gastroenteritis virus (TGEV) (Doyle and Hutchings, 1946), porcine epidemic diarrhea virus (PEDV) (Wood, 1977), and porcine deltacoronavirus (PDCoV) (Woo et al., 2012). Despite their differences on pathogenicity, PECs are clinically and histopathologically indistinguishable yet genetically and antigenically related (Saif et al., 2019). Therefore, differential diagnosis relies on laboratory methods (Gimenez-Lirola et al., 2017; Masuda et al., 2016). Simultaneous testing of multiple markers in a single reaction volume (sample) is especially relevant for the rapid identification of clinically and taxonomically related pathogens. The amino-

terminal receptor-binding (S1) portion of the S protein was identified as highly sensitive and specific antigen target for differential serodiagnosis of PorCoVs (Gimenez-Lirola et al., 2017). Thus, this study described the design and development of a parallel dot ELISA-like multiplex immunoassay (AgroDiag PorCoV) for simultaneous detection and differentiation of TGEV, PEDV and PDCoV antibody.

## 2. Material and methods

### 2.1. Experimental serum samples

Sixty 7-week-old pigs with not previous history of porcine coronavirus infections and pre-screened negative for different porcine coronaviruses were used in this study. Animals (12 per group) were

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experimentally inoculated with PEDV (USA/IN/2013/19338E), TGEV Purdue (ATCC VR-763), TGEV Miller (ATCC VR-1740), PDCoV (USA/IL/2014), or mock inoculated with Eagle's minimum essential medium (EMEM, ATCC) as previously described (Gimenez-Lirola et al., 2017). Serum samples ( $n = 480$ ) were collected from all groups on day post-inoculation (DPI) -7, 0, 7, 14, 21, 28, 35, and 42.

## 2.2. Generation of PEDV, TGEV and PDCoV recombinant S1 proteins

The coding region of the S1 domain derived from consensus sequences derived from PEDV, TGEV and PDCoV proteins were expressed in a mammalian expression system (pNPM5 expression vector and HEK293 cells), and the soluble Fc-S1 fused proteins were purified by protein A affinity chromatography (GE Healthcare) followed by Fc-tag cleavage and further purification by nickel (Ni)-chelating Sepharose Fast Flow affinity chromatography (GE Healthcare) as previously described for PEDV S1 protein (Gimenez-Lirola et al., 2017). Purified PEDV S1 (717 aa), TGEV S1 (771 aa) and PDCoV S1 (504 aa) proteins (Fig. S1) were dialyzed against phosphate-buffered saline (PBS) (Gibco®, Thermo Scientific) pH 7.4 and analyzed by SDS-PAGE and Western blot.

## 2.3. Chip manufacturing

Chip manufacturing processes were performed in a clean room environment (ISO 7) to prevent particle and biological contamination (Fig. 1A). Functionalization of 96-well high binding microplates (2592, Corning) was performed by coating a functionalized dextran layer to the bottom of the wells, specifically designed for the assay according to a patented method developed by Innobiochips (Melnyk et al., 2012). Affinity purified recombinant S1 proteins (TGEV, PEDV, PDCoV) and pig IgG (P100-105, Bethyl Laboratories), used as test positive control, were printed on the surface in triplicate to form an array of spots (spot

diameter  $\sim 130$  nm, Fig. 1A), using a sciFLEXARRAYER SX® (Scenion AG) automated liquid dispenser. Following proprietary blocking and stabilization steps, the plates were stored in a dry atmosphere at 20 °C until use.

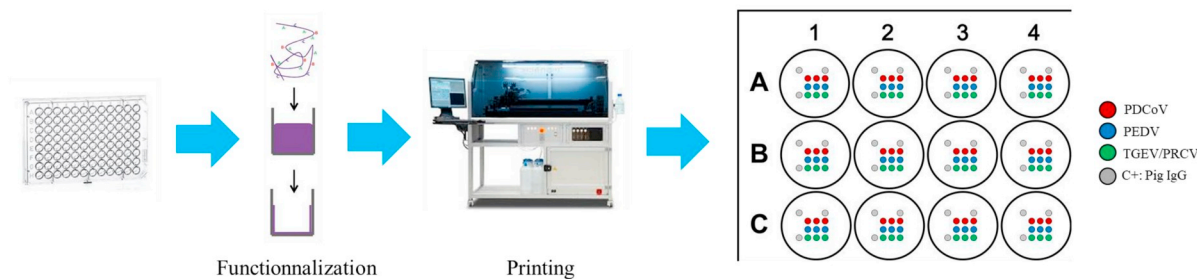
## 2.4. Assay protocol

Serum samples (100  $\mu$ L per well) were diluted 1:100 in a proprietary assay buffer and tested in duplicate (Fig. 1B). The plates were incubated 1 h at 37 °C on a microplate shaker (VWR International) at 300 rpm, and washed three times (200  $\mu$ L/well) with PBS pH 7.4, containing 0.1% of Tween 20 (Sigma). For conjugation, 100  $\mu$ L of proprietary conjugate buffer was added to each well, followed by 1 h incubation at 37 °C in the dark. After washing, 50  $\mu$ L of insoluble TMB (Calbiochem®, Merck) was added to each well and incubated for 15 min in the dark. After a final washing step with 200  $\mu$ L of mQ water per well, any trace of residual water was removed by incubation for 15 min at 37 °C. Images of individual wells were captured by a microplate reader (Clair®, Sensovation AG) operated by the software Sensospot® (Sensovation AG). The spot ("blue dots") mean intensity (MI) was calculated as the average pixel value inside a circle defining the spot perimeter (Fig. S2). The background MI was defined as the average pixel value in the local background outside the spot plus a margin so no signal from the spot was to be considered. The net signal intensity, correlated to the antibody responses, was calculated as the spot MI - background MI.

## 2.5. Data analysis

The MI cut-off values and associated diagnostic performance of each antigen target were determined by receiver operating characteristic (ROC) analysis (GraphPad Prism® 7). The diagnostic specificity was evaluated on negative samples ( $n = 240$ ) collected from PorCoV negative pigs. The diagnostic sensitivity ( $n = 191$ ; DPI 21 to 42) and

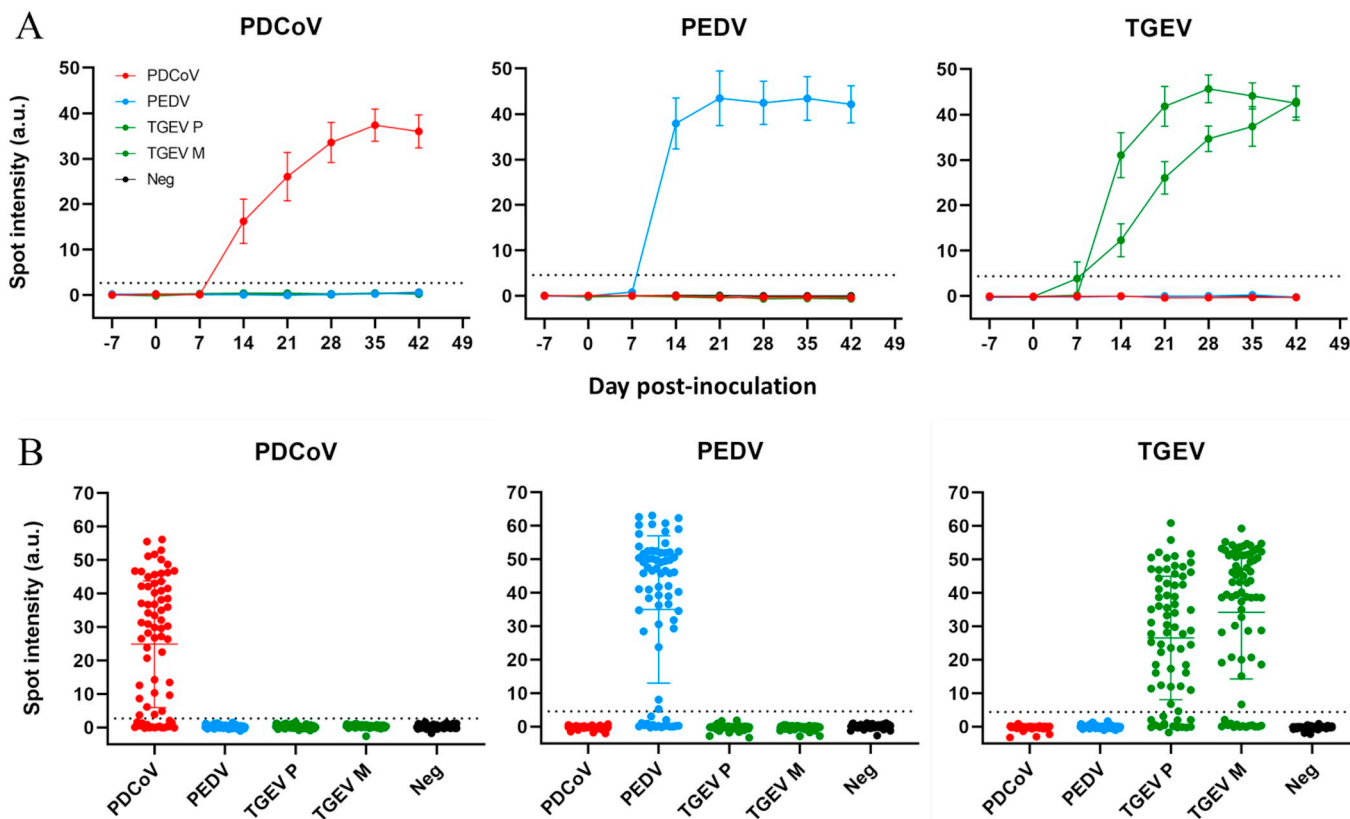
### A Chip manufacturing steps



### B User steps ~3h



**Fig. 1.** Overview of the design, fabrication and use of the AgroDiag porcine enteric coronavirus (PEC) multiplex planar immunoassay. (A) Manufacturing steps include functionalization of the wells of a microplate with a dextran layer before printing of the protein array in triplicate. (B) The assay protocol and working principle is similar to the solid-phase standard indirect ELISA. After the three typical incubation steps (serum sample, conjugate, and substrate), followed by microplate reading. The reaction was visualized as brown spots which intensity correlated with antibody levels to specific viral antigen target in the array. Four results are obtainable depending on incubation with a negative control, or samples from PDCoV, PEDV, TGEV inoculation groups. Scale bar corresponds to 1 mm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



**Fig. 2.** AgroDiag porcine enteric coronavirus (PEC) results. (A) AgroDiag porcine enteric coronavirus multiplex planar immunoassay spot intensity of serum IgG responses (means and standard errors), expressed as arbitrary units (a.u.), over time to each S1 protein antigen specific of porcine epidemic diarrhea virus (PEDV), transmissible gastroenteritis virus (TGEV) or porcine Deltacoronavirus (PDCoV) in pigs (12 per group) experimentally inoculated with PEDV (USA/IN/2013/19338E), TGEV Purdue (ATCC VR-763), TGEV Miller (ATCC VR-1740), PDCoV (USA/IL/2014), or mock inoculated with Eagle's minimum essential medium (EMEM, ATCC). (B) Distribution of cumulative AgroDiag PECs results obtained for each virus-specific S1 antigens (PEDV S1, TGEV S1, and PDCoV S1) on serum samples ( $n = 480$  total;  $n = 96$  per group) collected from pigs inoculated as described herein. Samples above the estimated cut-off (dash line) were considered positive.

**Table 1**

Detection of serum IgG antibody response (number of positive samples above selected cut-offs) among inoculation groups by day post inoculation using the AgroDiag porcine enteric coronavirus (PEC) multiplex immunoassay.

Inoculation group	Cut-offs	Day post-inoculation								
		-7	0	7	14	21	28	35	42	
PDCoV	2.69	0/12	0/12	0/12	7/12	11/12	12/12	12/12	12/12	12/12
PEDV	4.6	0/12	0/12	1/12	11/12	11/12	11/12	11/12	11/12	11/12
TGEV Purdue	2.78	0/12	0/12	1/12	9/12	12/12	12/12	11/11 <sup>a</sup>	12/12	12/12
TGEV Miller	4.39	0/12	0/12	0/12	11/12	12/12	12/12	12/12	12/12	12/12

<sup>a</sup> One missing sample (quantity not sufficient) for one animal within the TGEV Purdue group at day post-inoculation 35.

detection rate over time (DPI -7 to 42) for each antigen was evaluated on positive samples collected from homologous inoculation group. The analytical specificity of each antigen was evaluated on samples collected between DPI 7-42 DPI from heterologous inoculation groups. On-way ANOVA with Dunnett's correction was used for multiple comparisons with  $\alpha = 0.05$  (GraphPad Prism® 7). Specifically, we compare the antibody response between inoculation groups by day post inoculation for each antigen target.

### 3. Results

#### 3.1. Dynamics of the IgG serum antibody responses in PEDV, TGEV and PDCoV inoculated pigs

The IgG serum antibody response to individual antigens evaluated over time (DPI -7 to 42) within each inoculation group is presented in

Fig. 2A. The IgG response was detected at DPI 7 on most pigs showing seroconversion, increasing thereafter with significantly higher ( $p < .05$ ) antibody levels than the negative control group at DPI  $\geq 14$ . Likewise, for each given antigen, pairwise comparison showed statistical significant differences ( $p < .05$ ) at DPI  $\geq 14$  between the antibody response to homologous vs. heterologous inoculation group, showing an absence of antigenic cross reactivity among S1 PECs-specific proteins (Fig. 2B).

#### 3.2. Diagnostic performance of the multiplex AgroDiag PorCoV immunoassay

Antigen-specific cut-offs were selected to ensure 100% diagnostic and analytical specificity for each given antigen target. Selected cut-offs and target-specific detection rate with each inoculation group over time is given in Table 1. The overall diagnostic sensitivity was 92% (44/48

positives, 95% CI 98,100) for PEDV S1, 100% (95/95 positives, 95% CI 98, 100) for TGEV S1, and 98% (47/48 positives, 95% CI 97, 100) for PDCoV S1.

#### 4. Discussion

The evaluation of antibody levels to multiple pathogen-specific targets by ELISA involves performing several assay workflows in parallel, which is time consuming and increases the cost and risk of error. The ability to detect and measure antibody levels to specific pathogens in one sample makes multiplex technology particularly appealing in the setting of continuous monitoring and control of infectious diseases in commercial swine herds. Multiplex immunoassay formats can be divided in planar and microbead-based suspensions assays. The overall working principle remains the same as for singleplex immunoassay, as the quantity of captured analytes (i.e., antigens) stays correlated to its concentration in the sample. This study describes a multiplex planar immunoassay for simultaneous detection differential serodiagnosis of three major PECs (PEDV, TGEV, and PDCoV). The technology overall format and working principle is similar to those from the solid-phase standard ELISA. The miniaturization of multiple targets in microarrays reduce the consumption of target used for coating, as well as the amount of samples and reagent volumes. The increased output per sample also allows to speed up the testing and reduce costs. In the platform described herein, virus-specific S1 domains, previously identified as highly sensitive and specific antigen target for differential serodiagnosis of PECs, were used as target antigens (Gimenez-Lirola et al., 2017). The design of a specific functionalization layer allowed for the successful association of the antigens in an array, while preserving their conformation and functionality. The colorimetric detection is sensitive, as for a given concentration of antibody in the sample, the quantity of antibody captured on the spot per unit volume is generally higher than if distributed throughout a larger surface (Kingsmore, 2006; Tighe et al., 2015). The diagnostic performance of this assay was evaluated on known status samples. Altogether, the results of this study demonstrate that the AgroDiag PEC multiplex immunoassay is an efficient and reliable test for differential detection of PECs.

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#### Declaration of Competing Interest

Authors Rémi Malbec, Elisa Vandenkoornhuysen, and Christophe Audebert are employees of GD Biotech, while Vianney Souplet and Christophe Olivier are employees of Innobiochips.

#### References

- Doyle, L.P., Hutchings, L.M., 1946. A transmissible gastroenteritis in pigs. *J. Am. Vet. Med. Assoc.* 108, 257–259.
- Gimenez-Lirola, L.G., Zhang, J., Carrillo-Avila, J.A., Chen, Q., Magtoto, R., Poonsuk, K., Baum, D.H., Piñeyro, P., Zimmerman, J., 2017. Reactivity of porcine epidemic diarrhea virus structural proteins to antibodies against porcine enteric coronaviruses: diagnostic implications. *J. Clin. Microbiol.* 55, 1426–1436.
- Kingsmore, S.F., 2006. Multiplexed protein measurement: technologies and applications of protein and antibody arrays. *Nat. Rev. Drug Discov.* 5, 310–321.
- Masuda, T., Tsuchiaka, S., Ashiba, T., Yamasato, H., Fukunari, K., Omatsu, T., Furuya, T., Shirai, J., Mizutani, T., Nagai, M., 2016. Development of one-step real-time reverse transcriptase-PCR-based assays for the rapid and simultaneous detection of four viruses causing porcine diarrhea. *Jpn. J. Vet. Res.* 64, 5–14.
- Melnyk, O., Bernard, J.-P.G., Philippe, J., Souplet, V., Olivier, C., 2012. Method for Functionalizing Surfaces For Analyte Detection.
- Saif, L.J., Wang, Q., Vlasova, Jung, K., Xiao, S., 2019. Coronaviruses. In: *Diseases of Swine*. Wiley-Blackwell, pp. 488–523.
- Tighe, P.J., Ryder, R.R., Todd, I., Fairclough, L.C., 2015. ELISA in the multiplex era: potentials and pitfalls. *Proteomics Clin. Appl.* 9, 406–422.
- Woo, P.C.Y., Lau, S.K.P., Lam, C.S.F., Lau, C.C.Y., Tsang, A.K.L., Lau, J.H.N., Bai, R., Teng, J.L.L., Tsang, C.C.C., Wang, M., et al., 2012. Discovery of seven novel mammalian and avian coronaviruses in the genus deltacoronavirus supports bat coronaviruses as the gene source of alphacoronavirus and betacoronavirus and avian coronaviruses as the gene source of gammacoronavirus and deltacoronavirus. *J. Virol.* 86, 3995–4008.
- Wood, E., 1977. An apparently new syndrome of porcine epidemic diarrhoea. *Vet. Rec.* 100, 243–244.