A multiplex assay for the detection of antibodies to relevant swine pathogens in serum

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Summary

Livestock industry supports the livelihood of around 1.3 billion people in the world, with swine industry contributing with 30 % of total livestock production worldwide. To maintain and guarantee this production, a pivotal point according to the OIE is addressing potential biohazards. To control them, permanent sero-surveillance is crucial to achieve more focused veterinary public health intervention and prevention strategies, to break the chains of transmission, and to enable fast responses against outbreaks. Within this context, multiplex assays are powerful tools with the potential to simplify surveillance programs, since they reduce time, labour, and variability within analysis. In the present work, we developed a multiplex bead-based assay for the detection of specific antibodies to six relevant pathogens affecting swine: ASFV, CSFV, PRRSV, SIV, TB, and HEV. The most immunogenic target antigen of each pathogen was selected as the target protein to coat different microsphere regions in order to develop this multiplex assay. A total of 1544 serum samples from experimental infections as well as field samples were included in the analysis. The 6plex assay exhibited credible diagnostic parameters with sensitivities ranging from 87.0 % to 97.5 % and specificities ranging from 87.9 % to 100.0 %, demonstrating it to be a potential high throughput tool for surveillance of infectious diseases in swine.

Keywords

Multiplex assay, African and Classical Swine Fever, Tuberculosis, PRRSV, SIV, Hepatitis E

Introduction

Livestock industry contributes globally with about 20-40 % of agricultural gross domestic product, supporting completely or partially the livelihood of around 1.3 billion people worldwide. Moreover, livestock is the source of 34 % global food protein, but it is not equally distributed, and it is especially vital to the economies of developing countries, where food insecurity is an endemic concern (ILRI, 2020; FAO, 2018, 2020a; FAO, ILRI, & cirad, 2019). Among different livestock sectors, swine industry plays a crucial role, with more than 30 % of total livestock production worldwide (FAO 2020b, 2020c). To maintain and ensure these productions, the World Organisation for Animal Health (OIE), published a guide to good farming practises, where addressing biohazards is a pivotal point to guarantee the production at different levels and to improve biosecurity for both, animals and humans (FAO & OIE, 2009). During the last decades, productions have undergone intensification and globalization processes that led to the reduction in herd numbers with a huge increment in the number of animals per herd. This, in combination with the increase in movement of animals, feed and products derived from these industries, led to the spread of pathogens all over the world (VanderWaal & Deen, 2018). Through a timely and reliable diagnosis and an ongoing surveillance, useful knowledge is obtained to allow more focused veterinary public health intervention and prevention strategies, to break the chain of transmission, and to give faster responses against outbreaks, thereby minimizing the impact of infectious diseases (Riley & Blanton, 2018; Turlewicz-Podbielska, Włodarek, & Pomorska-Mól, 2020).

The most important diseases affecting animals are tracked globally by the OIE. Some of the most relevant pathogens affecting swine as described in the manual of diagnostic tests and vaccines for terrestrial animals are: African Swine Fever Virus (ASFV), Classical Swine Fever Virus (CSFV), Porcine Respiratory and Reproductive Syndrome Virus (PRRSV), and Swine Influenza Virus (SIV) (OIE, 2019a). African Swine Fever (ASF) is a high-impact contagious disease in swine caused by a complex DNA virus:

62 ASFV. The disease can run different courses depending on host factors and strain
63 virulence. Haemorrhagic signs and exceptionally high lethality accompany infections
64 with highly virulent ASFV strains. Antibodies are detectable early upon infection and
65 for long periods (without predicting disease outcome) (Dixon, Sun, & Roberts, 2019).
66 Similar in clinical and pathological presentation, Classical Swine Fever (CSF) is a
67 highly contagious disease caused by a small RNA virus, CSFV. Since both diseases can
68 be found simultaneously in different countries, laboratory tools are necessary for the
69 proper identification of the pathogen (Malik et al., 2020; Schulz, Staubach, & Blome,
70 2017). Porcine Respiratory and Reproductive Syndrome is considered one of the most
71 important swine diseases that is caused by PRRSV. The disease is characterized by
72 respiratory syndrome in young pigs, and reproductive failure in pregnant sows, leading
73 to substantial economic losses to the pig industry. Several genotypes have been
74 circulating since its appearance in the 1980s with different virulence rates, that led in
75 2016 to the differentiation of the two main genotypes (PRRSV-1 and PRRSV-2) into
76 two separated species (Lunney et al., 2016; Kuhn et al., 2016, Montaner-Tarbes, Del
77 Portillo, Montoya, & Fraile, 2019). Finally, Swine Influenza is a respiratory pathology
78 caused by Influenza A viruses, most commonly by the subtypes H1N1, H1N2 and
79 H3N2, and more recently a pandemic H1N1 (Simon et al., 2014). Swine Influenza is a
80 highly contagious infection, which, usually, moves quickly within a herd reaching
81 morbidity rates near to 100 %, but with low mortality rates and rapid recovery.
82 Economic losses related to SIV infection are related to retarded weight gain and to the
83 animals' weakening, which can lead to secondary bacterial infections complicating the
84 disease (Janke, 2014; OIE, 2009; Van Reeth & Vincent, 2019). In addition, infection of
85 pregnant sows with influenza A virus leads to secondary losses through abortions and
86 other reproductive problems (Gumbert et al., 2020).

87 On the other hand, some zoonotic diseases are of interest to the swine industry not only
88 for the effects on pigs but also due to the role pigs play in the transmission route of
89 zoonotic agents to humans, especially from wild reservoirs. Bovine Tuberculosis (TB)
90 is one of these important zoonotic diseases. TB is caused by the different members of
91 the *Mycobacterium tuberculosis* complex, the most prevalent bacteria of this group that
92 causes infection in pigs is *Mycobacterium bovis*, which can cause disease in animals
93 like cattle and swine, and it can be transmitted to humans. TB is widely spread over the
94 world. Its control in wild species is challenging and, despite efforts carried out, TB

95   keeps on being endemic in wild populations from many countries (Bailey, Crawshaw,
96   Smith, & Palgrave, 2013; Cano-Terriza et al., 2018; Cousins, 2001; Pesciaroli et al.,
97   2014). During the last decades, another infectious agent has gained attention among
98   swine populations: Hepatitis E Virus (HEV). HEV is a pathogen that can be transmitted
99   to humans and has been identified in different animal species. Nowadays, it is known to
100   cause a highly prevalent and emerging zoonotic disease, responsible for the 3.3 %
101   hepatitis deaths worldwide. Its impact in animals is not well-established, since not all
102   susceptible species have been identified and because, in many cases, the clinical signs
103   are undetectable, hindering the proper development of surveillance programs (WHO,
104   2019; Kenney, 2019; Sooryanarain & Meng, 2020).

105   Within this context, the present study aims to develop a multiplex assay for the
106   detection of antibodies against some of the most relevant pathogens affecting the swine
107   population. This kind of assay will offer some advantages over individual assays, such
108   as reduced time and sample volume as well as possible variability between independent
109   assays.

110   Bead-based multiplex assays (BBMAs), commonly known under the trade names
111   xMAP Technology or Luminex assays, are a powerful high throughput technology. This
112   platform uses coloured code polystyrene microspheres as the surface for the capture
113   molecule binding, and, by mixing different microspheres regions within a single plate
114   well, allows the detection of multiple analytes within a single sample run. This
115   technology combines fluorescent-dyed microspheres, a detection instrument based on
116   lasers read-out, digital signal processing, and an analysis software (Christopher-
117   Hennings et al., 2013; Graham, Chandler, & Dunbar, 2019). While BBMAs are widely
118   applied in human health, with the development of methods for drug discovery
119   (Komnatnyy, Nielsen, & Qvortrup, 2018), diseases diagnosis (Grignard et al., 2019; Lu
120   et al., 2005) and immune response characterization (Jones et al., 2002) among others,
121   work in the veterinary field has been more limited, although interest has been rising
122   recently (Chen et al., 2016; Fabian et al., 2020; Hoste et al.; Laamiri et al., 2016; Ragan
123   et al., 2018). To date, there are a few commercial assays available (References TRD-
124   500 and TRD-502, Biovet Inc. Saint-Hyacinthe, Canada). Moreover, previous studies
125   have shown that bead-based assays might be slightly more sensitive than ELISA
126   technology, and they open the chance not only to simultaneously test against several
127   pathogens, but also to differentially evaluate several antigens of a given pathogen in one

assay, which is of great interest for complex and variable microorganisms (Aira et al., 2019; Chen et al., 2013).

In the present work, the most immunogenic target antigens of the described pathogens have been obtained as recombinant proteins using different heterologous systems, and a 6plex assay for the differential detection of antibodies against these relevant swine pathologies has been developed.

Materials and methods

Antigens

The viral protein 30 (VP30) of ASFV (BA71 strain) was produced with a 6xHis tag in insect cells and the protein was further purified from the insoluble fraction under denaturing conditions (Aira et al., 2019). The MPB83 antigen of *Mycobacterium bovis* was expressed fused to GST in insect cells, and the protein was purified from culture media by affinity chromatography using a glutathione column (Cardoso-Toset et al., 2017). The nucleocapsid protein (N) of PRRSV-1 was obtained in *Escherichia coli* fused to the T7 phage capsid protein, and further purified from insoluble cell fraction under denaturing conditions (Rodriguez et al., 1997). The nucleoprotein (NP) of SIV was produced fused to a 6xHis tag in insect cells, and protein was further purified from culture media by affinity chromatography using a nickel column. The glycoprotein E2 of CSFV was produced in insect cells with a 6xHis tag and purified from the culture media by affinity chromatography with a copper column (Sastre et al., 2016). The p239 protein from HEV comprises a partial sequence of the capsid protein and was produced in *E. coli* fused to a 6xHis tag and it was purified by affinity chromatography using a nickel column.

Serum Samples

Reference serum samples for each pathogen were used for assay optimization. All sera were characterised as positive by the ELISAs used as the reference technique in this study. The ASFV-positive reference serum was provided by the European Union reference laboratory for ASF (EURL) and previously characterized by the OIE ELISA against the BA71 strain. The CSFV-positive reference serum was provided by the National and FAO reference laboratory for CSF at the Friedrich-Loeffler-Institute (FLI) and characterized by VNT (virus neutralization) against CSFV strain Alfort/187 with a

50 % neutralization dose (ND50). The PRRSV-positive reference serum was provided by INIA-CISA, it consisted on a pool of serum samples obtained from experimentally infected animals slaughtered at 84 days post-infection. The SIV-positive reference serum was obtained from a field animal vaccinated with the commercially available vaccine FLUSURE® PANDEMIC (Zoetis) and previously characterized by ELISA. The TB-positive reference serum was provided by the Unit of Prophylaxis and Control of Bacterial Zoonoses, High Institute of Health, Rome, Italy, and was obtained from a Nebrodi black pig grown in semi-freedom conditions previously evaluated for IFN-γ and antibodies by different ELISA formats. The Hepatitis E positive reference serum was obtained from a naturally infected pig and it was previously characterised by ELISA at FLI. The negative porcine serum was purchased from Gibco (reference. 26250084) obtained from a young pig (less than one year old) from New Zeeland and previously characterized by ELISA.

For further validation, four panels of well-characterized swine sera were included in the present study.

For detection of antibodies against ASFV, a panel of 181 serum samples from pigs used in vaccination/challenge experiments at BSL3 facilities at PIR, were included in this study (Sanchez-Cordon et al., 2018). Briefly, 29 pigs were immunized with an attenuated Benin strain and serum samples were collected at different days post infection (dpi). The animals were boosted 21 days later with the same virus and on day 40 they were challenged with virulent Benin 97/1. Moreover, a collection of 14 sera samples (13 positive and 1 negative sample) obtained in experimental infection experiments carried out at FLI facilities and used in German national ring trials were included in the assay.

For detection of antibodies against CSFV, 46 experimental serum samples from pigs infected at FLI facilities were used, that is routinely employed for batch release testing and assay validation (Sastre et al., 2016). Briefly, 23 positive samples collected from pigs experimentally infected with different strains of CSFV and 23 negative samples were included in the assay. Among the negative samples, two were obtained from non-infected animals, eleven were obtained from animals experimentally infected with CSFV but negative by ELISA (early phase of infection), and ten were obtained from

190 animals infected with other cross-reactive pestiviruses: Border disease virus (BDV) and
191 Bovine viral diarrhoea virus (BVDV).

192 For detection of antibodies against PRRSV, a collection of 180 field serum samples
193 obtained from pigs vaccinated and non-vaccinated against this virus in Spanish farms
194 (GST laboratory, Lleida) was included in this study.

195 For detection of antibodies against *M. bovis*, a collection of field samples obtained from
196 25 juveniles and 55 adults wild boar; and a collection of experimental samples obtained
197 from 9 non-infected wild boar and 51 sera from experimentally infected wild boards
198 were included in the assay (Fresco-Taboada et al., 2019). Moreover, a collection of 42
199 samples (positive and negative) obtained from pigs grown in semi-freedom was
200 included in the assay evaluation.

201 Finally, a collection of 941 field swine serum samples from German farms, provided by
202 IVD Gesellschaft für Innovative Veterinärdiagnostik mbH (IVD GmbH), and not
203 previously characterised for the diseases, were also included for the proper evaluation of
204 our assay.

205 Samples were classified into positive or negative, based on different commercial
206 ELISAs used as the reference technique in this study for the characterisation of serum
207 samples for statistical evaluation:

208 - INgezim PPA Compac (11.PPA.K3, Eurofins-Ingenasa) competition assay for
209   detection of specific antibodies against ASFV.
210 - IDEXX CSFV Ab Test (IDEXX) competition assay for detection of specific
211   antibodies against CSFV.
212 - INgezim PRRSV 2.0 (11.PR2.K1, Eurofins-Ingenasa) indirect assay for
213   detection of specific antibodies against PRRSV.
214 - INgezim Influenza Porcina (11.FLU.K1, Eurofins-Ingenasa) for detection of
215   specific antibodies against SIV.
216 - INgezim TB Porcine (11.TBP.K1, Eurofins-Ingenasa) indirect assay for
217   detection of specific antibodies against *M. bovis*.
218 - In-house ELISA developed at the FLI (Martin Eiden, personal communication)
219   indirect assay for detection of specific antibodies against HEV.

220 **Coupling of Target Antigens to Beads**

221 The viral target antigens were covalently coupled to different carboxylated magnetic
222 bead regions (Luminexcorp, Austin, USA) following manufacturer's indications.
223 Briefly, one million carboxylated magnetic microspheres, identified individually by a
224 unique fluorescence ratio (regions #15, #18, #20, #21, #25 and #34, MagPlex®
225 Microspheres, Luminex) were activated according to the NHS/EDC protocol
226 (Hermanson, 2013), based on a two-step carbodiimide reaction. Activated beads were
227 incubated with different amounts of the recombinant proteins, ranging from 2.5 to 25 μg
228 per one million beads, in a final incubation volume of 500 μl, and incubated for 2 h with
229 rotation in dark. After washing steps, the supernatant was replaced with 1 ml of storage
230 buffer (PBS, 1% BSA, 0.05% azide). Bead concentration after coupling was determined
231 by counting on a Neubauer plate. The coupled microspheres were kept in storage buffer
232 at 4°C in the dark until use, as recommended by manufacturer. The beads were used
233 within the next 3 months after coupling.

234 A coupling confirmation assay was performed using serial dilutions of monoclonal
235 specific antibodies to each protein: anti-6xHis tag (MA1-21315; Invitrogen, Carlsbad,
236 CA) for VP30 and NP, 83CA3 (Eurofins-Ingenasa, Madrid, Spain) for MPB83, 1AC7
237 (Eurofins-Ingenasa, Madrid, Spain) for N, and 14E11 (Eurofins-Ingenasa, Madrid,
238 Spain) for E2, in order to assess the coupling efficiency. P239 coupling efficiency was
239 directly tested against reference sera.

240 **Bead-Based Assay for Antibody Detection in Swine Serum**

241 To perform the 6plex assay, individual antigen-coupled microspheres were sonicated
242 and vortexed for homogenization. A microsphere mixture was prepared by mixing the
243 six bead regions in assay buffer (PBS, 3 % (w/v) Milk, 0.3 % (v/v) Tween20) to a final
244 concentration for each region of 25 beads/μl. Fifty microliters of this bead mixture were
245 added over 50 μl of individual pig serum samples diluted at 1/200 in assay buffer. The
246 mixture was incubated for 30 min at room temperature (RT) and 650 rpm in a shaker.
247 For this assay, 96-well plates (Stripwell™ Microplate Medium binding Polystyrene,
248 Costar) previously blocked for 15 min, were used. The plate was protected from light
249 during all the incubation process. After each incubation step, the plate was washed
250 twice with washing buffer (PBS, 0.3 % (v/v) Tween 20) using a magnetic washer. Each
251 well was incubated with 50 μl of anti-swine IgG monoclonal antibody 1BH7 (Eurofins-
252 Ingenasa, Madrid, Spain) labelled with biotin, at a final concentration of 4 μg/ml in
253 dilution buffer (PBS, 1 % (w/v) BSA, 0.05 % (v/v) Tween20), for 30 min at 650 rpm

254 and room temperature. Then, 50 μl/well of Streptavidin R-phycoerythrin (Molecular
255 probes®, life technologies) were added at a final concentration of 2 μg/ml in dilution
256 buffer and they were incubated for 30 min at 650 rpm and room temperature. The beads
257 were then resuspended in washing buffer and the results were read out in a Bio-Plex®
258 200 (Bio-Rad) or in a MAGPIX® dispositive (Luminexcorp, Austin, USA). The signal
259 was measured as median fluorescence intensity (MFI) of at least, 50 events of each bead
260 region.

261 Two wells per assay were incubated in the absence of sample, only with assay buffer, as
262 a blank signal, which was subtracted from the sample signal. Positive and negative
263 controls were included in all assays to confirm the performance of the test.

264 **Statistical Analysis**

265 Data were statistically analysed by a ROC curve analysis using the MedCalc® 10
266 software (MedCalc Software Ltd, Seoul, Republic of Korea) to establish the optimal cut
267 off value for each antigen and the performance characteristics of the multiplex assay.

268

269 **Results**

270 **Development and Optimization of the Multiplex Bead-Based Assay**

271 Initially, the coupling conditions were optimised for each of the selected target antigen.
272 The optimal amount of antigen to couple one million microspheres of the selected
273 regions, was selected as the minimum concentration of antigen that rendered the highest
274 signals when incubated with serial dilutions of its monoclonal antibody, as previously
275 described in materials and methods [Table 1].

276 Using the bead mixture, assay conditions were optimised to avoid cross-reactions, and
277 beads' reactivity was evaluated against reference sera. All microspheres optimised for
278 the multiplex assay, exhibited high MFI signals against their corresponding reference
279 serum with no cross-reactions between microspheres (data not shown).

280 **Analysis of Experimental and Field Sera with the Multiplex Assay**

281 Once the assay was optimised and the proper performance of reagents was confirmed, a
282 panel of experimental and field sera was evaluated by the 6plex assay. First, selected

283   groups of serum samples evaluated by the multiplex assay were classified as positive or

284   negative by the corresponding ELISA used as reference in the present study [Table 2].

285   For the determination of the multiplex diagnostic parameters, MFI for each microsphere

286   region was evaluated by a ROC curve assay, to determine the best cut off values to

287   obtain the greatest performance parameters [Figure 1]

288   For the detection of specific antibodies against ASFV, the developed assay exhibited a

289   100 % specificity and a 93.9 % sensitivity with a cut off value of 1162 [Figure 1A].

290   Among the 82 positive experimental samples included in the assay, 5 samples gave a

291   false negative result. And within the negative samples included in the evaluation of

292   ASFV diagnostic parameters (n=521), none false positive results were obtained [Table

293   3], indicating that the assay developed is highly accurate for application in field.

294   For the detection of specific antibodies against CSFV, the cut off was selected as 1126.

295   With that value, diagnostic parameters were established as a 91.3 % sensitivity and a

296   98.0 % specificity [Figure 1B]. In total, 603 samples were evaluated with the bead-

297   based assay and classified into positive or negative according to the ELISA used as

298   reference in the study. Among the samples classified as positive (24) only two false

299   negative samples were obtained from co-infections of CSFV with other cross-reactive

300   *Pestivirus* (BDV, BVDV) exhibiting a good sensitivity. On the other hand, within the

301   579 samples classified as negative with the ELISA used as reference, 8 samples gave a

302   positive signal with quite high values of MFI with the newly developed assay [Table 3].

303   These eight samples belonged to the group of sera obtained from experimental

304   infections with the CSFV: six sera were obtained at 21 days post-infection exhibiting

305   for all the samples signals above 2000 MFI; another serum was obtained at 96 days

306   post-infection and the last one at 926 days post-infection.

307   For the detection of antibodies against PRRSV-1, the assay developed exhibited a

308   sensitivity of 87.0 % and a specificity of 94.6 % for the optimal cut off value of 1970

309   MFI [Figure 1C]. Among the 1383 samples analysed with the multiplex assay, 756

310   samples were classified with the ELISA used a reference in the present study. Within

311   the 292 samples classified as positive, 254 gave a positive result with the bead-based

312   assay obtaining a total of 38 false negative results. Besides, among the 464 samples

313   classified as negative with the ELISA, 439 gave the same result with the Luminex

314   assay, obtaining only 25 false positive samples [Table 3]. It should be mentioned that,

315 among false positive samples, 8 did not give a value next to the cut off, they gave high
316 MFI signals (>5000).

317 For detection of antibodies against SIV, cut off was established according to the ROC
318 curve analysis, at 2091 MFI, showing a sensitivity of 95.8 % and a specificity of 87.9 %
319 [Figure 1D]. Among the 1383 serum samples analysed, 391 were evaluated with the
320 ELISA used as reference in the study. A total of 175 samples among the 199 samples
321 classified as negative, gave also a negative result with the multiplex bead-based assay,
322 with a total of 24 false positive samples. Whereas 184 samples among the 192 samples
323 classified as positive, gave a positive MFI signal in the bead-based assay, with 8 false
324 negative samples [Table 3].

325 The assay developed for detection of antibodies against *M. bovis* using the MPB83 as
326 target antigen exhibited great diagnostic parameters, with a sensitivity of 97.5 % and a
327 specificity of 99.8 % for the established cut off (MFI = 5043) [Figure 1E]. Among all
328 the samples classified as positive with the reference ELISA (n=120), 117 gave a
329 positive signal (over 5043) with the newly developed bead-based assay. And among the
330 rest of the sera samples tested (1264) classified as negative by the ELISA used as
331 reference or obtained from German farms (free of TB), only 3 gave a false positive
332 result, which was confirmed as false positive by ELISA [Table 3].

333 Finally, in the case of HEV, the diagnostic parameters obtained for specific antibodies
334 detection to P239 were 95.7 % sensitivity and 94.6 % specificity for an established cut
335 off value of 2602 [Figure 1F]. Among the 1001 samples analysed, 432 samples were
336 classified by the in-house ELISA previously developed for the detection of antibodies to
337 HEV. Within the 221 samples classified as negative with the ELISA used as reference,
338 12 false positive samples were obtained, as well as 9 false negatives within the 211
339 samples characterised as positive with the in-house ELISA [Table 3].

340 **Discussion**

341 Currently, the major limitation of diagnostic assays is that they only allow the detection
342 of one pathogen per run, hindering the evaluation of wide panels of diseases. In this
343 context, the optimization of multiplex assays could reduce the limitation of laboratory
344 diagnosis since they allow the detection of several pathogens differentially and
345 simultaneously, being applicable on complex syndromes with shared symptomatology,
346 as well as creating new opportunities for integrated surveillance programs. Apart from

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that, multiplex assays exhibit technical advantages: they require smaller sample volumes, they reduce labour and time, and they reduce the potential variability when compared to the performance of six individual ELISAs regarding sample handling, interassay variability, as well as reducing human error. Moreover, miniaturization of the assay surface may render to higher sensitivity (Arnold, Scobie, Priest, & Lammie, 2018; Elshal & McCoy, 2006; Ling, Ricks, & Lea, 2007). In this study, we developed a multiplex bead-based assay which could be used as a high throughput-screening tool to assess the presence of specific antibodies to six high-impact pathogens in swine with good diagnostic parameters.

The presence of antibodies in serum indicates the development of an immune response in an organism against a past exposure to a given pathogen or as a result of vaccination. Antibodies can be tracked for months to years since their appearance, giving information about past vaccination or pathogen exposure, and their presence can prevent the remerge of a given pathogen (Alter & Seder, 2020; Arnold et al., 2018).

In the case of ASFV and CSFV, the presence of antibodies in samples collected within the European Union is a direct indicator of a previous infection. For ASFV there are no available vaccines (OIE, 2019b) and, in the case of CSFV, vaccines have been developed and successfully employed for control of the disease. However, after its eradication in several countries, vaccination was prohibited so as to guarantee the proper surveillance of the disease, and its implementation is restricted to emergency cases where disease cannot be controlled by other methods (OIE, 2020). For the detection of antibodies to ASFV, good diagnostic parameters were obtained: Sn. 93.7 % and Sp. 100.0 %, whereas for CSFV both parameters were slightly lower with a Sn of 91.7 % and a Sp of 98.0 %. When deeply looking at the results obtained for the detection of antibodies to CSFV, we observed that the 8 false positive samples obtained with this multiplex assay belonged to experimentally infected animals. Briefly, 6 sera were obtained at 21 days post-infection, another one was obtained at 96 days post-infection, and the last one at 926 days post-infection. According to the rest of the samples analysed, during those periods of time, antibodies were detected in other animals, what could indicate that the newly developed assay may have greater sensitivity parameters than those first calculated. This superior sensitivity was previously reported for different pathologies when directly comparing some Luminex assays with the respective ELISAs (Aira et al., 2019; Chen et al., 2013).

In the case of PRRSV and SIV, there are some commercially available vaccines, so antibodies detection can be due to both scenarios, an indicator of a past infection or an indicator of successful vaccination programs (OIE, 2009). For detection of antibodies to PRRSV, a specificity of 94.6 %, with high MFI signals for some of the false positive samples, and a sensitivity of 87 % were obtained. The sensitivity parameter was the lowest obtained within the multiplex assay. PRRSV is a highly variable pathogen which has been divided in two different species according to their ORF7 (codifying for N protein) sequence: PRRSV-1 and PRRSV-2. Among these species, an identity of 59-63 % has been described for ORF7, having significant differences in protein sequence too (Dea, Gagnon, Mardassi, Pirzadeh, & Rogan, 2000). In the present study, we only included the protein sequence for PRRSV-1, the most prevalent species in Europe. However, PRRSV-2 is also circulating in Europe and it is used in some of the vaccines developed. The ELISA used as reference in the present study uses a chimeric protein for both species. Therefore, the low sensitivity observed in the multiplex assay may be improved by the introduction of the N protein from PRRSV-2 coupled to a different bead region, potentially allowing the differentiation of the species causing infection. Diagnostic parameters obtained in the multiplex assay for the SIV antibody detection, were determined as Sn 95.8 % and a Sp 87.9 %. These values could be explained by the diagnostic performance of the ELISA used as reference in this study, which, according to manufacturer's indications, has a Sn of 87 % and a Sp of 89 %. Thus, some of the false positive and false negative samples obtained with the multiplex bead-based assay may be explained by a misclassification obtained by the ELISA used as reference.

For tuberculosis control, no commercial vaccines are available, and detection of antibodies in these populations can be considered a direct indicator of infection. Moreover, Germany has been declared free of this disease, reason why all samples obtained from German farms were considered negative to antibodies. In these conditions, the detection of antibodies against TB within the multiplex bead-based assay developed exhibited great diagnostic parameters (Sn: 97.5 %; Sp: 99.8 %).

To our knowledge, no vaccines are available for swine against HEV, thus detection of antibodies is a direct indicator of a past infection. For detection of antibodies to HEV, the bead-based assay exhibited a Sn of 95.7 % and a Sp of 94.6 %. Since the same antigen was used for the coating of ELISA plates and coupling to the magnetic microspheres, differences in antibody detection may be explained by the different

detector molecule used in the two assays. While the ELISA uses protein G coupled to peroxidase as detector molecule, the bead-based assay uses an anti-swine-IgG antibody labelled to biotin, which is a more specific recognition molecule (Choe, Durgannavar, & Chung, 2016). Moreover, bead-based assays have shown to be slightly more sensitive than ELISA in some cases, detecting lower amounts of IgG in serum, what may be an explanation for the decrease in specificity of the multiplex assay when compared to the ELISA.

To sum up, we can conclude that the developed multiplex assay exhibited promising performance parameters, which can reliably determine the immune status of a herd against several relevant pathogens. The implementation of this diagnostic assay on high impact diseases as the ones described, may be advantageous for National Veterinary Authorities, simplifying the application of epidemiological studies to swine populations. The developed multiplex assay could be improved in further studies by the incorporation of new antigens from other relevant pathologies. As well, since microspheres are obtained independently, the assay could be customised including and excluding pathogens as desired.

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Conflict of interest statement

The authors declare that they have no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon request.

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Tables

Table 1. Coupling conditions for target antigens

Pathogen	Antigen	Coupling concentration (μg/10^6 beads)	Bead region
ASFV	6xHis-VP30	5	15
CSFV	6xHis-E2	2.5	25

PRRSV	P10-N	5	20
SIV	6xHis-NP	5	21
M. bovis	GST-MPB83	2.5	18
HEV	6xHis-P239	25	34

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Table 2. Serum samples analysed in the 6plex assay

Group of sera samples	Number of samples analysed with each bead region (number of samples characterised by the reference ELISA of the respective disease)					
	ASFV	**CSFV**	**PRRSV**	**SIV**	**TB**	**HEV**
Experimental ASFV samples	181 (181)	181 (181)	-	-	139 (139)	-
Experimental ASFV/CSFV samples (FLI)	60 (60)	60 (60)	60 (0)	60 (0)	43 (43)	60 (0)
Field samples for PRRSV	180 (180)	180 (180)	180 (180)	180 (0)	180 (25)	-
Experimental samples for TB	139 (139)	139 (139)	139 (83)	139 (0)	-	-
Field samples for TB	43 (43)	43 (43)	43 (26)	43 (0)	60 (0)	-
Field samples (IVD)	-	-	941 (467)	941 (391)	941 (468)	941 (432)
Total	603 (603)	603 (603)	1383 (756)	1383 (391)	1383 (675)	1001 (432)

Table 3. Correlation between multiplex assay and reference ELISA

Samples classification	Number of samples for each pathogen					
	ASFV	**CSFV**	**PRRSV**	**SIV**	**TB**	**HEV**
True positives	77	22	254	184	117	202
True negatives	521	571	439	175	554	209
False positives	0	8	25	24	1	12
False negatives	5	2	38	8	3	9
Total	603	603	756	391	675	432

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Figures

Figure 1. ROC curve analysis for the determination of optimal cut off values for specific antibodies detection to: **A.** VP30 (ASFV), **B.** E2 (CSFV), **C.** N (PRRSV), **D.** NP (SIV), **E.** MPB83 (*M. bovis*), and **F.** P239 (HEV). Y-axis shows the MFI values for each sample. X-axis shows the classification of samples into positive (1) and negative (0) according to ELISA used as reference.