Sensing and Bio-Sensing Research 27 (2020) 100315

Contents lists available at ScienceDirect

Sensing and Bio-Sensing Research

journal homepage: www.elsevier.com/locate/sbsr

Initial trail results of a magnetic biosensor for the rapid detection of Porcine Reproductive and Respiratory Virus (PRRSV) infection

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ARTICLE INFO

Keywords:
Porcine Reproductive and Respiratory virus
Resonant Coil Magnetometer
Magneto-immunoassay
Competitive immunoassay
Serology
Pig serum

ABSTRACT

The resonant coil magnetometer quantifies paramagnetic particles (PMPs) and has been used to develop magneto-immunoassays in a range of formats. The advantage of magneto-immunoassays is that they are relatively inexpensive, portable, easy to perform and give results in under 5 min. Porcine Reproductive and Respiratory Virus (PRRSV) is an infection of domesticated pigs producing large economic losses in the swine industry current diagnosis is performed using commercially available ELISA kits. Here we describe the development of a competitive magneto-immunoassay (MIA) and pilot study with porcine serum samples. The data show that this technology has the potential for use as a rapid and portable in field system for the detection of antibodies in porcine serum to PRRSV. A range of assay parameters and magnetometer settings were optimised, including the concentration of antibody conjugated PMPs used in the assay and movement of an external magnet to pull particles to a sensor surface. PRRSV positive control serum demonstrated competition with antibody conjugated PMPs with a dose dependent relationship. The magneto-immunoassay developed showed good agreement with the PRRS IDEXX X3 ELISA. The PRRSV magneto-immunoassay demonstrated a sensitivity of 73% and specificity of 100%. The results suggest that a rapid assay using the magnetometer technology detects specific anti-PRRSV antibody in pig serum. The magneto-immunoassay is suitable for use as a rapid 'on-site' method for the serological detection of PRRSV infection.

1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV), is an infection of domesticated pigs producing large economic losses in the swine industry worldwide and is currently considered to be the most economically important infectious disease that is faced by the swine industry. PRRSV is a small enveloped, single-stranded positive-sense RNA virus approximately 50–65 nm in diameter and belongs to the family *Arteriviridae*. The genome of PRRSV is approximately 15 kb in length and is comprised of at least ten open reading frames (Orfs) [1–3]. PRRSV consists of two species: PRRSV-1 isolates are of European origin while PRRSV-2 originated in North America, the disease was first observed in the 1980's [4,5] and is now prevalent throughout the US, Europe and Asia. In the United States PRRSV infection results in losses of over $664 million annually [6–8]. In Europe estimates of losses have been made using a disease model and could be up to €650,090 per farm [8]. PRRSV-1 and PRRSV-2 share ~60% identity at the nucleotide level, in addition, each species can be further divided into several clades or substrains [9]. The key consequence of PRRSV infection is a respiratory disease in piglets characterized by fever, lethargy, and failure to thrive frequently developing into interstitial pneumonia. Other effects of PRRSV infection include abortion, weak piglets, and mummification together with stillbirth. The effects of PRRSV infection result in elevated mortality amongst piglets [10,11]. Infection of adult pig's particularly male boars however is largely asymptomatic, but flu-like symptoms are observed in the finishing phase affecting weight.

Current control measures involve detecting infected individual pigs and farms using commercially available ELISA kits e.g. IDEXX PRRSV X3™ to detect the presence of antibodies to PRRSV, followed by vaccination. The IDEXX PRRSV X3™ ELISA is an indirect immunoassay utilising peptide fragments of the highly immunogenic PRRSV nucleoprotein (N), also known as open reading frame protein 7 (Orf7) as the antigen. PRRSV Orf7 is highly immunogenic and induces early antibody responses in pigs [12]. PRRS ELISA assays are the mainstay of pig

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https://doi.org/10.1016/j.sbsr.2019.100315
Received 10 July 2019; Received in revised form 29 November 2019; Accepted 2 December 2019

testing programmes, however there are several limitations of ELISA assays. ELISA assays are performed in laboratories by specialist staff, the assays require several incubation and wash steps and take several hours to complete. In the UK, porcine blood samples are collected by a veterinary surgeon and transported to a laboratory for analysis by ELISA. This can cause a delay between sample collection and reporting of results and therefore a delay in the implementation of control measures. In the study described paramagnetic particles (PMPs) are used as a label in immunoassays. The PMPs are quantified using a device the Resonant Coil Magnetometer (RCM), [13], (Patent, WO 2008/114025). The advantages of this technology are that the assay matrix has little effect on the assay measurements, it is relatively inexpensive, gives results within minutes and is easy to use and therefore has the potential for development for use onsite by non-technical individuals.

The aim of this investigation is to show the development of a magneto-immunoassay to detect antibodies to PRRSV in porcine serum and the potential for use of this technology for rapid, cost effective "on-site" diagnosis of PRRSV.

2. Materials and methods

2.1. Serum samples

IDEXX X3 tested frozen porcine serum samples (n = 22) taken during normal veterinary surveillance were supplied by Clarity BioSolutions and were kept at −20 °C until testing.

2.2. PRRSV Orf7

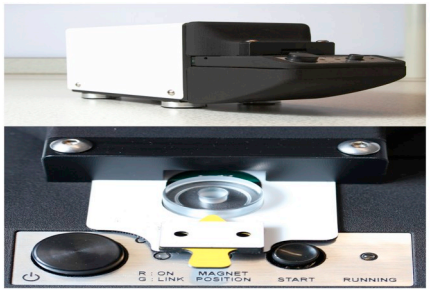
The Orf7 was kindly provided by Professor Vladimir Celer (University of Veterinary and Pharmaceutical Sciences, Brno, CZ) which was produced by expressing and purifying the PRRSV Orf7 sequence of the Lelystad strain of PRRSV in *E.Coli*. The recombinant PRRSV Orf7 included an N-terminal x6 His Tag. Orf7 was prepared immediately prior to use by centrifugation at 10,000 ×*g* to sediment any protein aggregates, the protein concentration of the supernatant was determined using the Bicinchoninic Acid assay (BCA assay), ThermoFisher Scientific, (Loughborough, UK).

2.3. Magnetometer and assay cuvettes

Instead of an enzyme label and colorimetric read out as in ELISA, PMPs are detected by virtue of their magnetic properties by a device, the resonant coil magnetometer (RCM), Fig. 1. PMP numbers correlate directly with the voltage output of the RCM [13]. Magneto-immunoassays have been developed in a range of immunoassays formats [14–16]. This is the first study where a magneto-immunoassay has been developed for serology. A diagrammatic representation of the PRRSV Magneto-immunoassay is shown in Fig. 2.

The RCM was developed at the University of the West of England and a prototype instrument produced by Clarity BioSolutions Ltd. in collaboration with Sarum Scientific Ltd. to take individual assay polystyrene cuvettes (Fig. 1). The assay cuvettes manufactured by Boddingtons Plastics Ltd., Tonbridge, UK were 15.9 mm diameter clear orientated Polyflex polystyrene discs which had a 5 mm central hole. The base of the well in the cuvette was formed by the attachment of 100 μm thick polystyrene film (Sidaplex, La Giraud, France) using 3M9485 pressure sensitive adhesive (3M PLC, 3M Centre, Bracknell, UK). This thin polystyrene base formed the sensor surface and was positioned directly above the resonant coil of the magnetometer which had a 3 mm spiral coil.

When the assay cuvette was placed in the magnetometer the external magnet moves into position under the resonant coil for 1 min to pull particles to the sensor surface. After this time the external magnet retracts and non-bound particles diffuse away from the surface. An individual result is taken as the difference in millivolts of the data trace at points A and B as shown in Fig. 1.



a. Data trace showing an increase in voltage when the external magnet moves in and out in relation to the magnetometer coil.
b. The Magnetic dwell time of the external magnet and effect on the baseline before and after approach if paramagnetic particles are present (b) or not (a)

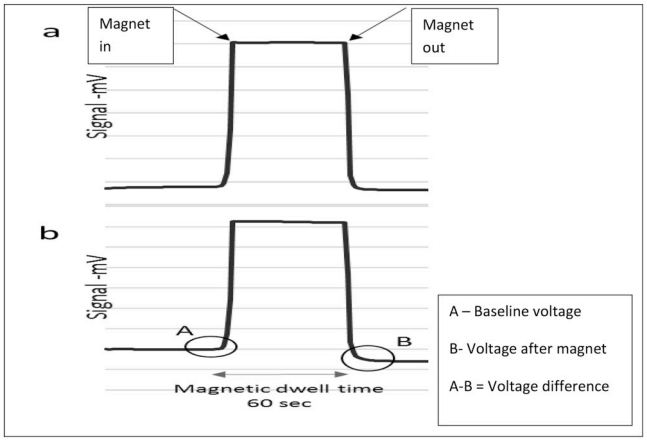


Fig. 1. Magnetometer and polystyrene consumable in position.

a. Data trace showing an increase in voltage when the external magnet moves in and out in relation to the magnetometer coil ie. the "magnetic dwell" time.
b. The magnetic dwell time of the external magnet and effect on the baseline before and after approach if paramagnetic particles are absent (a) or present (b).

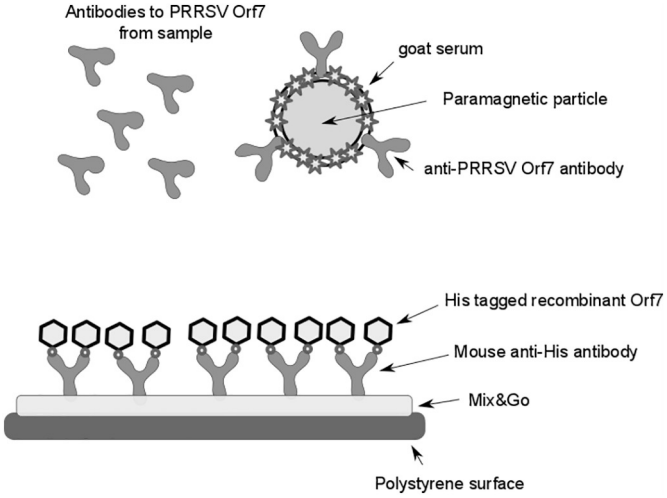


Fig. 2. Diagram to show the principle of the competitive magneto-immunoassay for anti-PRRSV antibodies

2.4. Preparation of paramagnetic particles

Dynal Tosylated 1 μm paramagnetic particles (PMPs), Invitrogen, Paisley, Scotland were coated with anti-PRRSV Orf7 nucleoprotein

(SDOW17A -Rural Technologies Inc., South Dakota, USA), a mouse monoclonal antibody, using 0.1 M borate buffer pH 9.5, at 37 °C for 1 h as described in the manufacturer's conjugation protocol. Prior to conjugation the antibody was mixed with normal goat serum or bovine serum albumin (BSA), (Sigma-Aldrich, Poole, UK) to give anti-PRRSV antibody concentrations of 0%, 0.3%, 1.25% and 5% of the total amount of protein. Forty migrogram protein/mg beads was conjugated to the particles. Following addition of protein the PMPs were sonicated for 4 × 30s pulses with 60s intervals at a setting of 10 μm using a Soniprep 150 sonicator (MSE, Lower Sydenham, UK) and again at 3.5 h post conjugation to ensure the dispersal of any PMP aggregates formed during conjugation. PMPs were stored at 4 °C in 500 μl of storage buffer (0.1% bovine serum albumin (BSA, Probumin, EMD Millipore UK Limited, Dundee, Scotland) and 0.05% Tween 20 (Sigma-Aldrich, Poole, UK) in phosphate buffered saline (PBS) until needed. The coated PMPs were used for up to 4 weeks post conjugation.

Confirmation of antibody conjugation to the PMP was carried by determining the quantity of anti-PRRSV Orf7 immobilized on the PMPs using a secondary antibody with a horse radish peroxidase (HRP) label. Briefly, 5 μl of PMPs (adjusted to 1×10^{10} PMP/ml) was added to 500 μl of a 1:2000 dilution of rabbit anti-mouse/HRP antibody (Dako, Cambridge, UK) in dilution buffer (1% BSA, 0.05% Tween 20 in PBS). The tube was incubated for 1 h at 37 °C on a Dynal rotator. Following incubation the PMPs were washed three times with 500 μl of wash buffer (0.1% BSA, 0.05% Tween 20 in PBS). The PMPs were vortexed well after each wash to re-suspend the PMPs. After the final wash the PMPs were transferred to a fresh tube and re-suspended in 100 μl of wash buffer. Three 10 μl aliquots of suspended PMPs were transferred into fresh tubes to which 100 μl of the HRP substrate, Tetra Methyl Benzidine (TMB), ThermoFisher Scientific, Loughborough, UK) was added. The PMPs were mixed and incubated at room temperature for 4 min before 100 μl of 1 M H_2SO_4 stop solution was added. The PMPs were removed from the supernatant with the aid of a DynaMag™ magnet (ThermoFisher Scientific, Paisley, UK). The supernatant was transferred into a well of a 96 well micro-titre plate and the absorbance measured at 450 nm on a microplate reader (Anthos II, Anthos Labtec Instruments GmbH, Austria).

The effect of the antibody loading in a magnetic assay was investigated using assay cuvettes coated with PRRSV Orf7 at a concentration of 5 μg/ml, as described below. One hundred microlitre of PMPs prepared as described containing 5×10^7 antibody conjugated PMPs was added to each assay cuvette and inserted into the magnetometer to record the assay trace.

2.5. Sensor surface preparation

The polystyrene sensor surface was first activated using proprietary binding reagent (PBR), (Mix&Go, Anteo Diagnostics, Brisbane, Australia). The recombinant PRRSV Orf7 which contains a x6 His Tag at the N-terminal was immobilized on the sensor surface via a mouse anti-HIS antibody (Life Technologies, Paisley, Scotland).

To activate the polystyrene surface, 40 μl of a proprietary binding reagent was added to an assay cuvette and incubated for 1 h at room temperature. The PBR was removed and the assay cuvettes washed twice with 250 μl of PBS and once with 250 μl of deionised water. Then 50 μl of mouse anti-His antibody (1/100 or 1/500 in PBS) was added to the cuvette and incubated for 1 h at 37 °C after which each assay cuvette was washed three times with 250 μl of wash buffer (0.05% Tween 20 in PBS). Finally the sensor surface was blocked by adding 200 μl of 2.5% BSA in PBS and incubating overnight at 4 °C.

After incubation each cuvette was washed three times with 250 μl of PBS/0.05% Tween 20 and 50 μl of PRRSV Orf7 solution diluted in PBS (concentrations of: 0, 10, 20 and 40 μg/ml) was added. After incubation at 37 °C for 1 h in a humidified atmosphere each consumable was washed three times with 250 μl of PBS + 0.05% Tween 20 to remove unbound PRRSV Orf7 and then stored in 200 μl of PBS/1%BSA/0.02% sodium azide until use.

To demonstrate PRRSV on the sensor surface, 100 μl mouse monoclonal anti – PRRSV-Orf7, diluted to 500 ng/ml in dilution buffer was added to cuvettes and incubated at 37 °C for 1 h. Antibody was removed and all wells were washed four times in wash buffer, then 50 μl of rabbit anti mouse Ig-HRP conjugate (Dako Limited, Ely, UK) diluted 1/1000 in dilution buffer was added to each cuvette and incubated for 1 h at room temperature. The antibody was removed and the cuvette washed four times in wash buffer followed by the addition of 50 μl of TMB substrate (Sigma-Aldrich, Poole, UK). After a 5 min incubation at room temperature 50 μl of 1 M H_2SO_4 stop solution was added and the plate read at an absorbance of 450 nm on a microplate reader.

The effect of PRRSV Orf7 loading on the sensor surface in a magneto-immunoassay was investigated using assay cuvettes coated with PRRSV Orf7 at concentrations of: 0, 10, 20 and 40 μg/ml as described above. Ten microlitre of PMPs (containing 5×10^7 PMPs with a 1.25% antibody loading) was added to each assay cuvette and inserted into the magnetometer to record the assay trace.

2.6. Validation of sensor surface by ELISA (Confirmation that antibody conjugated PMPs bind to the PRRSV ORF7 coated consumable surface)

PRRSV Orf7 coated consumables were prepared and blocked as described above. Porcine PRRSV antibody positive serum (LSI-EU) or PRRSV antibody negative serum, (MRI negative) both from LSIVet™, Life Technologies Corporation, Lissieu, France, was added to each consumable. Sera were diluted in assay buffer (1/200, 1/400, 1/600, 1/800) and 100 μl of diluted sera or assay buffer added. The consumables were incubated for 1, 15, 30 and 60 min and then the sera were removed by washing such that only Orf7 bound antibodies were detectable by enzyme immunoassay as described in Section 2.5 above.

2.7. PRRSV Magneto-immunoassay

Anti-PRRSV antibody coated PMPs (1.25% anti-PRRSV antibody in goat serum) were diluted in assay buffer (1% BSA, 0.2% PEG6000 (Sigma-Aldrich, Poole, UK) in PBS) to achieve a concentration of 5×10^8 PMPs/ml and sonicated 3 × 20 s pulses with the sonicator set to an amplitude of 10 μm.

Experiments with PRRSV antibody positive (Porcine PRRS/EU-Serum, LSI-EU) and a PRRSV antibody negative, (MRI negative) control sera both from LSIVet™, (Life Technologies Corporation, Lissieu, France) were performed to demonstrate the magneto-immunoassay. Four concentrations of PMPs were tested containing 0.5×10^7, 1.0×10^7 and 2.0×10^7 antibody conjugated PMPs. Here, 50 μl of assay buffer or 50 μl of serum was added to an eppendorf tube containing 100 μl of PMPs containing the PMP numbers described, the cuvette was washed 4× with assay buffer. Then 150 μl of PMP/serum or PMP/assay buffer was added to each assay cuvette and placed in the magnetometer to obtain the data trace. For magneto-immunoassays with test sera, PMPs were diluted prior to use such that a 150 μl volume contained 0.5×10^7 PMPs. One hundred and fifty microlitre of PMPs was mixed with 50 μl of test serum or assay buffer for the blank, and added to the assay cuvette, coated with 5 μg/ml Orf7 as described above. The consumable was then placed immediately into the magnetometer and the measurement cycle started. Results were expressed as a ratio of the test sample (S) sera voltage change (defined above) and the no serum, termed blank (B) thereafter voltage change.

2.8. Sample testing

A pilot study in the PRRSV magneto-immunoassay was performed on 22 serum samples from pigs that were identified as being positive or negative for PRRSV antibodies using the commercial IDEXX PRRS X3 ELISA kit (IDEXX Laboratories, NL). The ELISA tests were performed

according to the manufactures instructions and results read using an Anthos II plate reader (Anthos Labtec Instruments GmbH, Austria) at 550 nm. The results were calculated according to the manufactures instructions where a positive result was defined when the ratio of the sample absorbance/positive control absorbance (after subtraction of the background control from each) was > 0.4. For the Magneto-immunoassay the mean voltage change from triplicate samples was determined and the sample voltage change/positive control voltage change determined. A positive result was determined using the S/B result for 1/400 and 1/2000 dilutions of the LSI positive control in the magneto-immunoassay, i.e. average S/B ratio of the two results. The dilutions of the LSI positive control chosen are always positive and negative respectively in the IDEXX PRRS X3 assay (n = 3).

3. Data analysis

All assays were performed in triplicate and the Mean and Standard error of the mean (SEM) were determined. The S/P ratio of each sample in the PRRS IDEXX X3 ELISA and the S/B ratio of each sample in the PRRSV Magneto-immunoassay were calculated using mean values. Positive and negative PRRSV samples were determined in the PRRS IDEXX X3 ELISA according to the manufacturer's instructions where any sample with an S/P ratio is > 0.4 is positive. In the PRRSV Magneto-immunoassays a cut-off value was determined using the mean of the MIA results for the LSI-EU control tested at 1/800 and 1/2000 dilutions, which are positive and negative in the PRRS IDEXX X3 ELISA respectively.

The Sensitivity, Specificity, Positive Predictive Value (PPV) and Negative Predictive Value (NPV) for the Magneto-immunoassay were determined by comparing the number of positive and negative samples detected in relation to the PRRS IDEXX X3 assay.

4. Results

4.1. Assay development

4.1.1. Use of Mix&Go to activate the consumable surface

The base of each consumable is comprised of 100 μm thick polystyrene film. The polystyrene in ELISA plates is ɣ- irradiated and results in the formation of a charged surface, such that charged molecules e.g. proteins will attach to the surface through many hydrogen bonds. Polystyrene film is uncharged and therefore to use this substrate in an immunoassay the surface needs to be functionalised or charged. In this study we achieved this by activating the surface with Mix&Go a commercially available Activation Reagent where metal polymers allow binding to negatively charged residues on proteins https://www.anteotech.com/. The success of this pre-treatment to increase PRRSV Orf7 binding to the polystyrene substrate can be seen in Fig. 3. The use of Mix&Go gave up to a 3-fold enhancement of the activity of the sensor compared with preparation of the surface with PBS. This demonstrates the effectiveness of polymeric metal ions in the product to attach proteins to synthetic surfaces.

4.1.2. Improved interaction of PRRSV Orf7 and anti-PRRSV antibody through pre-adsorption of anti-HIS antibody

PRRSV Orf7 is a recombinant antigen that has an N-terminal x6 HIS tag, this feature was used to ensure that PRRSV Orf7 was specifically bound to the polystyrene substrate by the prior addition of anti-HIS antibody. The antigen bound will also all be orientated in the same way which could improve interaction with anti-PRRSV Orf7 antibody. This was tested by comparing the amount of PRRSV-Orf7 detected plus and minus anti-HIS antibody. The data in Fig. 4 shows the comparison of two concentrations of anti-HIS antibody 1:100 and 1:500 with a range of concentrations of PRRSV Orf7 5-40 μg/ml. At the 1:100 dilution of anti-HIS antibody an increase in the amount of antigen detected was observed across all antigen concentrations. This occurred either through increased amount of PRRSV Orf7 binding or improved orientation of the antigen to improve interaction with antibody. This concentration was used in all future experiments.

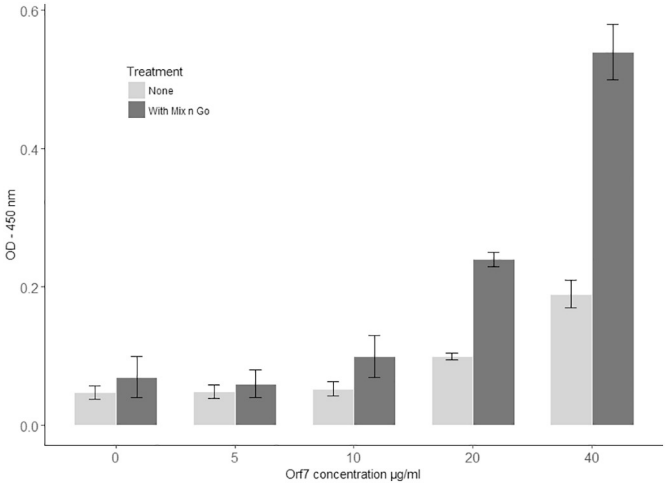


Fig. 3. Graph to show the effect of Mix & Go on the binding of PRRSV Orf7 antigen to the consumable surface.

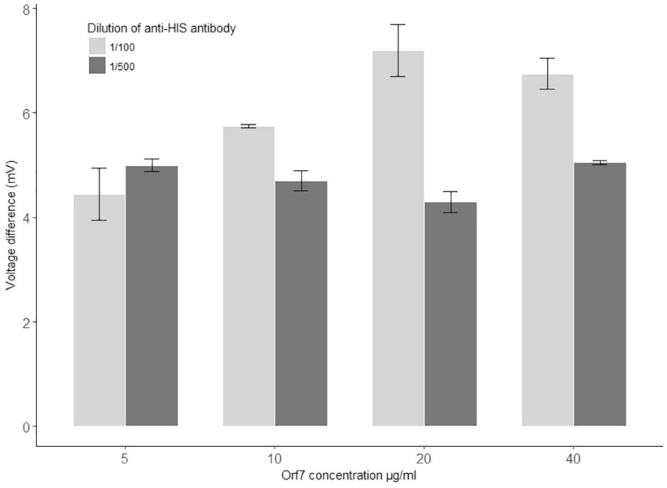


Fig. 4. Effect of anti-HIS antibody concentration on the interaction of anti-PRRSV antibody conjugated PMPs with PRRSV Orf7 coated consumables.

4.1.3. Evaluation of the optimum antibody concentration and blocking agents used to conjugate PMPs

The amount of antibody present on the PMPs is an important factor in developing an immunoassay, as this determines the total number of antibody binding sites that will be available in the competitive assay. Experiments were performed to demonstrate the effect of anti-PRRSV antibody loading on the PMPs and the loading of PRRSV Orf7 on the sensor surface. The immune-enzymatic assay reflected the total amount of antibody immobilized to the particles, which increases as the antibody loading increased as expected as seen in Fig. 5. However the variability in the amount of antibody loaded also increases with the antibody loading concentration, this could potentially lead to unwanted variability in magneto-immunoassay results. In addition the error bars of the 1.5% and 5% preparations overlapped indicating that the total amount of antibody possible was saturating. For this reason PMPs were conjugated at a concentration of 1.5% in future experiments.

Magnetometer measurements in magneto-immunoassays are detecting the number of PMPs binding to the antigen coated consumable surface. In an experiment with increasing antibody loading 0.3–5% of total protein, the number of particles used per cuvette was 5×10^7

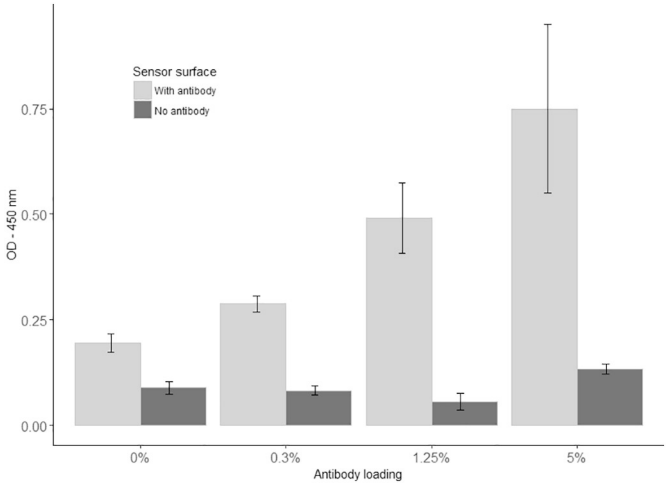


Fig. 5. Binding of PMPs plus or minus antibody conjugation to PRRSV Orf7 coated consumable surface ELISA.

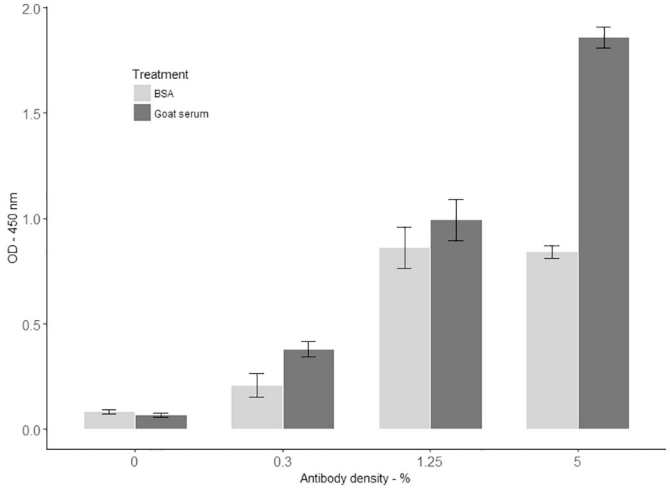


Fig. 7. Optimisation of blocking agent for preparation of PMP antibody conjugate

particles, the results are shown in Fig. 6. An antibody loading of 1.25% antibody gave the highest signal which corresponded to the best signal to noise ratio of the ELISA experiment. There are a greater number of antibody molecules on the surface of the particles with 5% antibody loading and it would be expected that this would generated the highest number of particles binding to the surface. However in these experiments the highest antibody loading of 5% did not correspond to the highest signal generated. The higher antibody concentration may have produced a reduced signal, due to steric hindrance or other mechanism resulting in a loss of antibody availability on the particles for binding to the antigen.

A number of blocking agents were investigated for use in PMP conjugation (data not shown). The best two were normal goat serum and bovine serum albumin see Fig. 7. The use of normal goat serum to dilute antibody for coating paramagnetic particles resulted in higher amounts of antibody binding to the particles compared to when bovine serum albumin was used to dilute the antibody across a range of antibody concentrations. This difference was particularly noticeable at a 5% antibody concentration, where the use of goat serum resulted in over twice as much antibody being immobilized on the particles. Normal goat serum was therefore used to prepare all PMP preparations in future experiments.

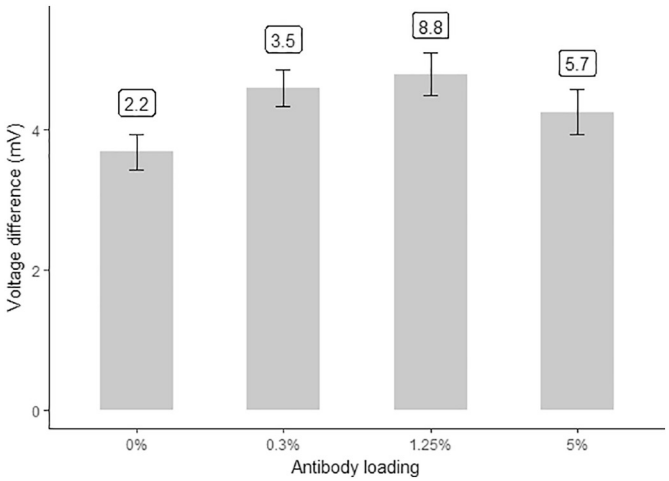


Fig. 6. Effect of anti-PRRSV antibody concentration used during conjugation on voltage difference in the PRRSV magneto-immunoassay

4.1.4. Evaluation of time of contact of PRRSV Orf7 antigen and PRRSV positive and negative porcine antibodies by ELISA

An experiment was performed to investigate the binding of PRRSV antibody positive and negative control sera (LSI-EU PRRSV positive and MRI PRRSV negative) to PRRSV Orf7 antigen. Bound antibodies were detected by anti- porcine- HRP tagged antibody.

As might be expected, the results in Fig. 8. show that increasing the incubation time of serum with PRRSV Orf7 coated sensor surfaces from 1 to 60 min, increased the amount of antibody binding to the surface. It would be expected that PRRSV Orf7 negative sera would not bind to PRRSV Orf7 antigen. Indeed results of the negative sample gave lower readings than the no antigen control after a 1 min incubation. At 15 min the negative sample at 1/200 and 1/400 dilutions gave results equal to the no antigen control. However as the length of time of incubation increased up to 60 min, the signal from the negative sample increased steadily to give readings greater than the no antigen control. These results suggests that low affinity, non-specific antibodies present in the serum are binding to the sensor surface which would result in high background readings. At 1 min incubation it is primarily high affinity antibody binding to the sensor surface and indicates that immunoassays

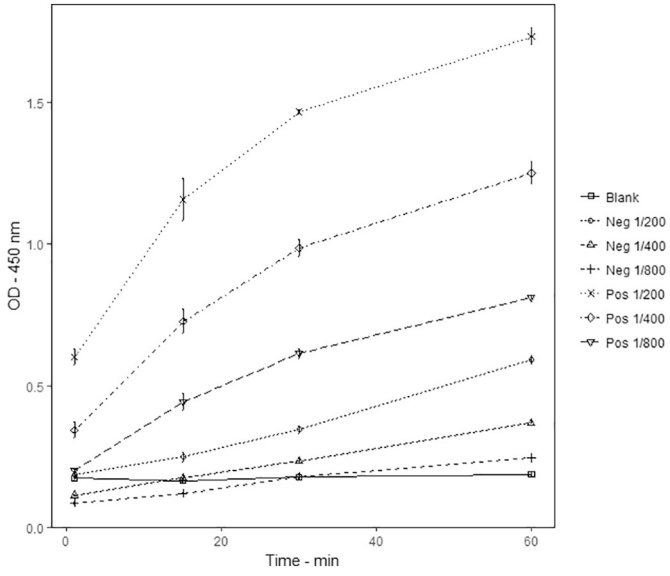


Fig. 8. Graph to show binding of anti-PRRSV Orf7 antibody to a PRRSV Orf7 coated consumable surface by ELISA incubated with a range of dilutions of PRRSV antibody positive and negative sera, at four time points.

performed and read with short incubation times approximately 1 min should have a lower background values.

It has been shown previously that pulling particles onto a sensor surface by the magnetometer dramatically reduces assay times [17]. These results suggest that a short magnetometer cycle would capture high affinity antibodies in the sample and less non-specific ones. Evaluation of the PRRSV magneto-immunoassay with a magnetic dwell time of 1–5 min showed that the optimum period for the external magnet to be in position below the sensing coli was 1 min. These results are consistent with the data presented above and therefore a magnetic dwell time of 1 min was in future experiments.

4.1.5. Evaluation of particle number for use in the PRRSV MIA

The importance of particle number in a magneto-immunoassay was demonstrated using commercially available PRRSV positive and negative serum samples. In a competitive magneto-immunoassay PRRSV positive samples contain antibodies to PRRSV that bind to the antigen on the sensor surface, thus preventing anti-PRRSV antibody conjugated particles from binding to the surface.

Experiments were performed to evaluate the PMP concentration to use in the Competitive magneto - immunoassay. In these experiments anti-PRRSV antibody coated particles at three dilutions were mixed with a 1/50 dilution of the serum sample and added to the assay cuvette. On commencement of the magnetometer cycle, particles are rapidly pulled to the surface and binding takes place between the antibody and the surface antigen and immobilises the particle to the surface which reduces the resonant frequency of the coil, while unbound particles diffuse from the surface when the magnetic field is removed and have little effect on the coil. An example of the results obtained is shown in Fig. 9, the signal from the cuvettes with no PRRSV Orf7 on the surface is due solely to paramagnetic particles present in solution above the sensor and within the magnetic field, with voltage difference increasing as the number of particles increases.

It can also be seen in Fig. 9 that when using 0.5×10^7 particles, the signal is greater for both the positive serum and negative serum, indicating that binding is taking place on the sensor surface. Here there is a lower response in the test with a positive serum sample compared to the result of a negative sample, as would be expected due to the competitive nature of the assay. When higher numbers of particles are used in the assay this differential between the positive and negative samples is lost. When 2×10^7 particles were used there was little difference between the positive serum, the negative serum and the blank as the mass of magnetic material is saturating the resonant coil and there is a large excess of paramagnetic particles which prevent diffusion of the unbound particles from the surface. This large mass of unbound particles is also detected by the coil masking the signal from specific interactions on the sensor surface. A similar result is observed when 1×10^7 particles are used in the assay but the magnitude of the measurements are reduced. This demonstrates the critical effect that the particle number has on the assay design. Ideally, when there is a number of particles that cover the surface with a monolayer all the particles will be captured. Adding a competitive antibody as found in a positive serum sample will reduce the number of particles that can bind to the surface. In this study 0.5×10^7 particles were used in all assays on serum samples.

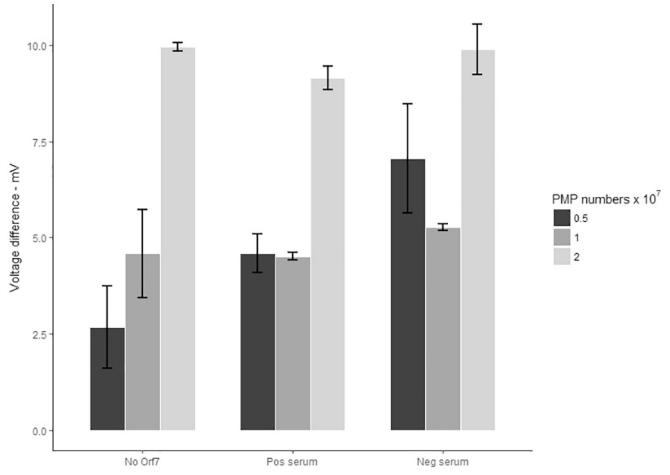


Fig. 9. Effect of particle number on competition in the PRRSV magneto- immunoassay by PRRSV antibody positive and negative control serum.

4.2. PRRSV Magneto-immunoassay results

4.2.1. Evaluation of PRRSV positive and negative control sera in the PRRSV magneto-immunoassay and determination of the assay cut-off value

The magneto-immunoassay measured the difference in the base line of the magnetometer trace before and after the first magnetic "pull-down" of particles on to the sensor surface. In the case of a PRRSV positive sample there are antibodies in the sample that compete with the antibody on the particle surface for the antigen immobilized on the sensor surface resulting in fewer particles binding to the surface and results in a lower response by the magnetometer. The mean voltage change results from the magneto-immunoassay are calculated from the ratio of the sample result/blank result. The blank was the assay in which there was no serum sample added and the maximum number of particles bind to the sensor surface. In this study the cut-off value for a positive sample was determined from the MIA mean S/B ratio of the PRRSV LSI-EU positive control S/B ratio at 1/800 and 1/2000 dilutions i.e. PRRS IDEXX X3 positive and negative dilutions of the positive control, the S/B cut-off using these values was determined to be of 1.06 see Table 1 below.

4.2.2. Evaluation of PRRSV positive and negative porcine serum samples (n = 22) by PRRSV magneto-immunoassay and PRRS X3 IDEXX ELISA

To demonstrate the utility of the magneto-immunoassay a pilot study to test 22 PRRS IDEXX X3 confirmed positive and negative serum samples in the magneto-immunoassay in triplicate was performed. The ELISA test defines the test result as being the ratio of the sample absorbance and the absorbance of the positive control supplied in the assay kit. A test result > 0.4 was defined as being positive. This test is the mainstay of pig testing programmes.

The PRRSV MIA PRRSV positive and negative assay results were determined from the sample S/B ratios. These are displayed in Table 2 alongside the PRRS IDEXX X3 assay results.

Using these results the sensitivity, specificity and PPV and NPV results for the PRRSV MIA were calculated according to Yerushalmy [18,19] see Table 3 below.

The mean PRRSV Magneto-immunoassay S/B ratio of all IDEXX confirmed positive and negative porcine sera tested is shown in Fig. 10. This cut-off has clearly separated two groups within the data, IDEXX confirmed positives mean S/B ratio $-1.164 +/- 0.08$ and IDEXX X3 confirmed negatives S/B ratio $-0.830 +/- 0.04$. The lower S/B ratio

Table 1
PRRSV MIA results for a range of dilutions of LSI-EU PRRSV positive control serum and determination of the PRRSV MIA cut-off value.

Sample (S)	Mean change in voltage (mV) n = 3	Standard error	PRRSV MIA S/B ratio	PRRS IDEXX X3 positive
LSI 1 in 200	5.03	0.783	1.378	P
LSI 1 in 400	4.53	0.207	1.241	P
LSI 1 in 800	4.10	0.367	1.123	P
LSI 1 in 2000	3.60	0.205	0.986	N
Blank (B)	3.65	0.152	1.000	NR

Key – Blank – PRRSV Orf 7 (5 μg/ml).
MIA Cut-off value – $1.123 \pm 0.986/2 = 1.06$.

Table 2
Comparison of PRRSV Magneto-immunoassay S/B ratios with IDEXX X3 S/P ratios.

Serum sample	PRRSV MIA S/B	Result cut-off 1.06	PRRS X3 IDEXX S/P	Result cut-off < 0.4
1	1.256	P	1.35	P
2	0.874	N	< 0.4	N
3	1.297	P	1.46	P
4	1.166	P	2.06	P
5	0.894	N	1.75	P
6	0.842	N	< 0.4	N
7	1.368	P	1.18	P
8	1.000	N	< 0.4	N
9	1.605	P	2.11	P
10	0.789	N	< 0.4	N
11	1.105	P	2.08	P
12	1.052	N	< 0.4	N
13	1.184	P	1.83	P
14	0.710	N	< 0.4	N
15	0.984	N	< 0.4	N
16	1.343	P	1.2	P
17	0.702	N	< 0.4	N
18	0.783	N	< 0.4	N
19	0.783	N	1.47	P
20	0.797	N	1.02	P
21	0.594	N	< 0.4	N
22	0.790	N	< 0.4	N
Results	True positive – 8	True negative – 11	False positive – 0	False negative – 3

Table 3
Summary results of the PRRSV Magneto-immunoassay in comparison to the PRRS IDEXX X3 ELISA.

Samples	Sensitivity	Specificity	PPV	NPV
Porcine serum N = 22	73%	100%	100%	79%

range for confirmed positives was 1.084 which is comparable with the cut-off determined using the PRRSV LSI-EU control positive at 1/800 and 1/2000 dilutions. Comparing the data from the PRRSV Magneto-immunoassay with the PRRS IDEXX X3 Gold standard, shows good agreement between the two assays. The PRRSV Magneto-immunoassay demonstrated a sensitivity of 73% and a PPV of 79% and specificity of 100% with a negative predictive value of 100%.

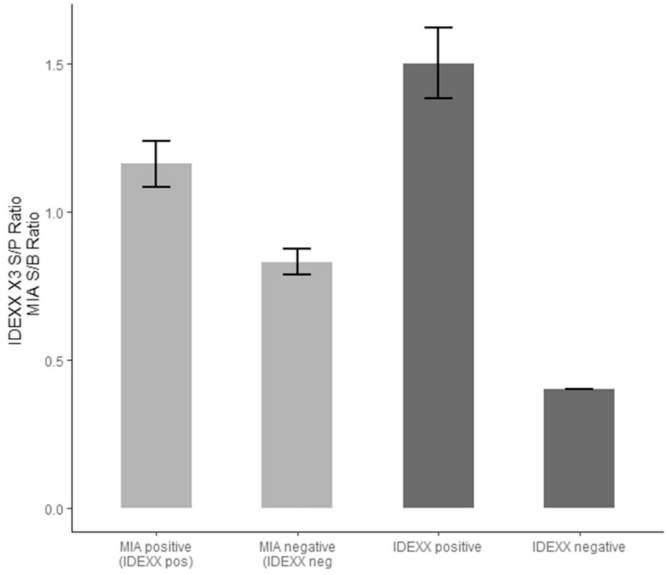


Fig. 10. Comparison of IDEXX X3 S/P ratio results with MIA S/B ratio results for IDEXX X3 confirmed PRRSV antibody positive and negative serum samples.

5. Discussion

Rapid diagnosis of Porcine Reproductive and Respiratory Virus (PRRSV) infection is essential to enable timely action to prevent spread of the infection. Current surveillance involves taking blood samples which are then sent to a central laboratory for serology, i.e. the detection of specific anti-PRRSV antibodies by ELISA. Several ELISA based assays are available commercially for PRRSV serology and have been compared for differences in specificity and diagnostic sensitivity [20]. The results showed significant differences in specificity and diagnostic sensitivity between the compared kits. These differences in the performance were particularly significant on PRRSV-negative farms, or farms with PRRSV stable sow herds. Therefore some ELISAs gave results that did not accurately detect the infection status in specific age groups. In addition ELISA assays require specialist equipment and personnel to perform the assays. Samples can deteriorate during transportation to a central laboratory and results are only reported after several days.

Magneto-immunoassays have been developed in other immunoassay formats and sample types, the assays are very rapid giving results in minutes and are less prone to interference from matrix effects than other types of immunoassays. In this study we developed a competitive magneto-immunoassay, for the detection of specific PRRSV antibodies using commercially available PRRSV control sera LSI and MRI. PMP number, antibody loading and PMP blocking agent all influenced the sensitivity of the magneto-immunoassay, but the largest impact on sensitivity was the length of time the external magnet was applied before magnetometer readings were taken. Very short magnet application enabled the competition between specific and high affinity anti-PRRSV antibody present in the serum and anti-PRRSV antibody present on the PMPs for PRRSV Orf7 (PRRSV capsid protein) present on the sensor surface to be observed. Longer application of the external magnet under the sensor resulted in non-specific interference of low affinity antibody present in the serum. Comparison of the data obtained with the same samples in the PRRSV Magneto-immunoassay and PRRS IDEXX X3 ELISA showed good agreement between the two assays. Improvement of the sensitivity of the Magneto-immunoassay by minor adjustment of PMP number and the magnetic dwell time are possible. Further work with a larger sample size of field samples will be necessary to validate the PRRSV magneto-immunoassay and cut-off determination with fresh serum samples.

6. Conclusions

This study is the first published magneto-immunoassay developed for the serological diagnosis of infection with this device. The magneto-immunoassay gives results in minutes, the assay and magnetometer are portable and are relatively inexpensive and can be operated by unskilled personnel.

Existing ELISA based serological diagnostics are often confounded by analytical interference e.g. non-specific interactions by heterophilic antibodies present in serum giving a high false positive rates [21]. The second version of the IDEXX PRRSV assay, the IDEXX PRRSV X2 assay using full length PRRSV Orf7 as antigen detected a relatively high rate of false positives [22]. In IDEXX PRRSV X3 the full length sequence of PRRSV Orf7 was replaced with peptide fragments of PRRSV Orf7, eliminating regions of the protein that were highly susceptible to interacting with substances causing analytical interference. In the magneto-immunoassay described we have used the full length sequence of PRRSV Orf7 and have found that this assay is less susceptible to interference. We suggest that this occurs due to reduction in incubation time from hours to minutes, leading to high affinity specific anti-PRRSV antibodies interacting with the antigen.

We have shown in a pilot study that the magneto-immunoassay results for IDEXX X3 positive and negative field serum samples were in good agreement with results from the gold standard assay. False negatives could be eliminated in the future by using fresh serum samples

rather than freeze thawed and further modifications of the antibody concentration, PMP concentration and magnetic “dwell time”. Larger numbers of field samples from a range of farming situations tested in parallel with the PRRSV IDEXX X3 assay will be performed as part of future work to validate the PRRSV magneto-immunoassay. In addition the performance of the PRRSV magneto-immunoassay will be evaluated with porcine saliva samples as these have been shown to be an alternative sample to use with PRRSV ELISA's [23]. The advantage of using saliva is non-invasive and therefore a veterinary surgeon is not essential to obtain the sample. The anti-PRRSV antibody magneto-immunoassay shows promise for ‘on farm’ rapid, cost effective and accurate diagnosis of PRRSV infection in farmed pigs. Use of this assay would prevent sample deterioration due to delay between sample collection and assay at a central lab. The immediate availability of results would enable rapid action to be taken by veterinary surgeons to control the spread of PRSSV infection. This technology could be applied to other PRRSV antigens e.g. non-structural protein 7 (Nsp7), [24] and in the serological detection of other infections in a wide range of species. The RCM has the potential to be developed as a multi-analyte testing platform.

Declaration of competing interest

The authors declare that they have no competing interests.

Acknowledgements

The authors wish to acknowledge Sarum Scientific Limited, Wiltshire for building the magnetometers, Professor Vladimir Celer (University of Veterinary and Pharmaceutical Sciences, Brno, CZ) and Mr. Autumn Teo (Placement student, Biomedical Science Department, Singapore Polytechnic) for his technical assistance.

This work was a collaborative project between Clarity Biosolutions, Ltd., Salisbury, UK and the Institute of Biosensing Technology (IBST), UWE, Bristol, UK to develop a novel detection system for animal diseases and zoonoses funded by Innovate UK, Project number 101466. Development of magnetometer immunoassay (MIA) technology for the rapid and cost effective detection of endemic animal diseases and pathogens (zoonoses) in a decentralised, point of use, setting'.

Availability of data and materials

All data supporting our findings is contained within the manuscript and the supplementary material.

Authors' contributions

RL, JK, DW and HBD designed the study. JB, ST and BM designed and performed the immunoassay experiments. JB and RL drafted the manuscript. All authors read and approved the final manuscript.

Consent for publication

Not applicable.

Ethics approval and consent to participate

The porcine serum samples were obtained during routine veterinary screening and tested in the IDEXX X3 assay.

Author contribution statement

We encourage you to submit an author statement file outlining all authors' individual contributions, using the relevant CRediT roles: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Software; Supervision; Validation; Visualization; Roles/Writing – original draft; Writing – review & editing. Please format with author name first followed by the CRediT roles: for an example and more details see authorship of a paper section here.

Jacqueline M Barnett writing - original draft, wrote, reviewed and edited the manuscript, Richard Luxton wrote, reviewed and edited the manuscript. Funding acquisition - David West, Hugh Ballantine-Dykes, Janice Kiely and Richard Luxton conceptualized the project and won the grant from Innovate UK. Project administration - David West, Hugh Ballantine-Dykes. Equipment design and validation - Patrick Wraith and Janice Kiely. Investigation - Jacqueline M Barnett, Bertrand M Monnier, Sue Tyler and Ed Regan performed, developed, organised and the experimental work. Data curation; Formal analysis - Jacqueline M Barnett, Richard Luxton and David West. Supervision - Jacqueline M Barnett, David West and Richard Luxton.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sbsr.2019.100315.

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