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A novel double recognition enzyme-linked immunosorbent assay based on the nucleocapsid protein for early detection of European porcine reproductive and respiratory syndrome virus infection

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Precise and rapid detection of porcine reproductive respiratory syndrome virus (PRRSV) infection in swine farms is critical. Improvement of control procedures, such as testing incoming gilt and surveillance of seronegative herds requires more rapid and sensitive methods. However, standard serological techniques detect mainly IgG antibodies. A double recognition enzyme-linked immunosorbent assay (DR-ELISA) was developed for detection of antibodies specific to European PRRSV. This new assay can recognize both IgM and IgG antibodies to PRSSV which might be useful for detecting in routine surveillance assays pigs that are in the very early stages of infection and missed by conventional assays detecting only IgG antibodies. DR-ELISA is based on the double recognition of antigen by antibody. In this study, the recombinant nucleocapsid protein (N) of PRRSV was used both as the coating and the enzyme-conjugated antigen. To evaluate the sensitivity of the assay at early stages of the infection, sera from 69 pigs infected with PRRSV were collected during successive days post infection (pi) and tested. While standard methods showed low sensitivity rates before day 14 pi, DR-ELISA detected 88.4% seropositive samples at day 7 showing greater sensitivity at early stages of the infection. Further studies were carried out to assess the efficiency of the new assay, and the results showed DR-ELISA to be a sensitive and accurate method for early diagnosis of EU-PRRSV infection.

1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is an enveloped, single-stranded positive-sense RNA virus that belongs to the family *Arteriviridae*, order *Nidovirales* (Cavanagh, 1997). Viral isolates have been classified in two different genotypes: type 1 PPRSV (European-PPRSV) and type 2 (US-PRRSV) (Nelson et al., 1993). PRRSV has been reported as a significant cause of swine morbidity and mortality worldwide (Albina, 1997). Consequently, the swine industry has been affected dramatically by PPRSV, suffering major economic losses every year (Neumann et al., 2005).

The viral genome is approximately 15 kb in length and contains nine known open reading frames (ORFs); ORF7 encodes the conserved nucleocapsid protein (N) (Mounir et al., 1995). The N protein is a small, basic, multi-functional protein with a molecular weight of 15 kDa, and has been identified as the most abundant and immunogenic viral protein (Nelson et al., 1994; Wootton et al., 1998). Such features make the N protein a suitable antigen for analysis of the immune response to PRRSV infection.

After PRRSV infection, pigs develop both antibody and cell-mediated immune responses. The IgM antibodies are detected first at day 5 post infection (pi) reaching a maximum peak at day 7 pi, and then declining rapidly to low levels by day 21 pi. The IgGs are detected first 7–10 days pi. After this period, IgG levels remain constant for months and decline finally to low levels by 300 days pi (Batista et al., 2004; Joo et al., 1997; Mulupuri et al., 2008).

Standard ELISAs are used widely for routine and experimental serodiagnosis of PRRSV (Brown et al., 2009; Chu et al., 2009; Diaz et al., 2005; Sorensen et al., 1997; Takikawa et al., 1996). However, these assays detect mainly IgG antibodies and have low sensitivity for IgMs, thus detection of infected pigs is not possible until day 7 pi. Prompt detection of PRRSV in swine farms is critical in order to carry out effective PRRS control, which could decrease the economic loss associated with an outbreak.

The aim of this study was to develop and optimize a rapid and sensitive assay to recognize not only IgGs but also other immunoglobulins, such as IgMs, allowing for early detection of EU-PRRSV infection. To accomplish this aim, PRRSV recombinant N protein was expressed in *Escherichia coli* and used as the coated and enzyme-conjugated antigen in a double recognition enzyme-linked

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immunosorbent assay (DR-ELISA). Distinct sets of sera obtained from field samples and from pigs infected experimentally were used to validate the new assay. The results of this study showed that DR-ELISA is a rapid and accurate method for early detection of EU-PRRSV infection.

2. Materials and methods

2.1. Viruses and cells

PRRSV isolates were grown in alveolar macrophages as described previously (Plana Duran et al., 1997).

E. coli strains DH5 and XL1-blue were used as the host for initial cloning of the target DNA into pET-3x vectors (Studier et al., 1990). *E. coli* BL21(DE3)-pLysS was used as the host cells for expression of the recombinant N protein (Grodberg and Dunn, 1988).

2.2. Serum specimens

2.2.1. Experimental samples

A collection of 378 serum samples was obtained from four different experimental vaccine trials carried out in a collaborative project with Fort Dodge Veterinaria SA Vall de Bianya (Girona, Spain). A total of 63 pigs, vaccinated with an experimental recombinant vaccine containing only the protein GP5, were bled at days 0, 4, 7, 14, 21 and 28 pi.

Sera from 6 pigs infected experimentally were collected weekly until day 56 pi. This trial was carried out in collaboration with the Centro de Investigación en Sanidad Animal (CISA)-Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA) Valdeolmos (Madrid, Spain).

2.2.2. Field samples

A total of 1002 field serum samples were collected in Spanish farms from pigs with no clinical symptoms and identified as seronegative by other commercial ELISAs.

Finally, a collection of 311 field samples was obtained from Spanish farms where PRRSV is endemic. These sera were expected to be PRRSV seropositive and this was confirmed by other commercial assays.

All animal work performed in this study meets animal welfare and ethical rules in the European Union which are in agreement with the American Physiological Society's guiding principles on the care and use of animals for research purposes.

2.2.3. Control samples

A commercial serum (Gibco USA) was used as the negative control in all the assays.

Serum from a pig immunized with the N recombinant was used as the positive control in all the assays.

2.3. Cloning and expression of the PRRSV N protein

The recombinant N protein of PRRSV (European strain, Olot/91 isolate) was expressed using the *E. coli* as described previously (Rodriguez et al., 1997). Purification of the expressed recombinant protein was done by FPLC using an ion-exchange column HiTrap Q HP (GE Healthcare), yielding purity greater than 90%. The presence of the p10-N protein was confirmed by SDS-PAGE. Finally the p10-N protein was labeled with peroxidase according to the method described by Nakane and Kawaoi (1974).

2.4. Enzyme-linked immunosorbent assays (ELISAs)

2.4.1. Double recognition ELISA (DR-ELISA)

European PRRSV's recombinant p10-N fusion protein (2.5 μg/ml) was used to coat 96 well microtiter plates (Maxisorp, Nunc) and incubated overnight (ON) at 4 °C in phosphate buffered saline (PBS) pH 7.4. After incubation, the wells were thoroughly washed using 0.05% Tween20 in PBS (PBST) and blocked with 1% BSA in PBS (PBS–BSA) for 1 h at room temperature (RT). For sample detection, 80 μl of HRP-conjugated antigen diluted in a predetermined volume of PBST was added to each well followed by 20 μl of serum in a one-step incubation. A negative and a positive control were included in each plate. After incubation for 60 min at RT, followed by extensive washing, the plates were incubated with the substrate (TMB-MAX, Neogen Corporation, Lexington KY) and the reaction was stopped by addition of 0.5 M sulfuric acid. The absorbance was measured at 450 nm in a Multiscan Ascent ELISA reader.

Statistical analyses were performed using MedCalc version 10.1.7.0 for Windows (MedCalc Software, Mariakerke, Belgium).

2.4.2. Indirect-ELISA (Ingezim PRRS Europa, Ingenasa)

The assays were performed according to the manufacturers' specifications.

2.4.3. Blocking-ELISA (Ingezim PRRS Compac, Ingenasa)

The assays were performed according to the manufacturers' specifications.

2.4.4. Capture ELISA for specific detection of anti PRRSV IgGs and IgMs

A 10 μg/ml concentration of monoclonal antibody specific for swine IgG or IgM in PBS was used to coat the 96 well microtiter plates. After incubation ON at 4 °C, plates were washed and blocked as described above. Test sera were incubated at a 1:100 dilution in 7.5 mM PBST-EDTA for 1 h at 37 °C. After a 3-step wash with PBST, European p10-N recombinant fusion protein at 2 μg/ml in PBST-EDTA was added. Samples were incubated for 30 min at 37 °C and washed subsequently three times. A predetermined dilution in PBST of monoclonal antibody specific for the N protein of European PRRSV and conjugated with peroxidase was added and incubated for 30 min at 37 °C. After a further extensive washing, the plates were incubated for 10 min with TMB and the reaction was stopped by the addition of 0.5 M sulfuric acid. The absorbance was measured at 450 nm in a Multiscan Ascent ELISA reader.

2.4.5. Commercial ELISAs (HerdChek PRRS 2XR and X3; Idexx Laboratories)

The assays were performed according to the manufacturers' specifications.

3. Results

3.1. Cloning and expression of the PRRSV nucleocapsid protein (N)

The complete ORF7 coding sequence of PRRSV was amplified by PCR from the Olot/91 isolate (European strain) with specific primers and cloned into pET-3x vector (Rodriguez et al., 1997). The PRRSV N protein was expressed in *E. coli* and was purified by FPLC. Analysis of the highly purified protein by SDS-PAGE followed by Coomassie blue staining revealed a 42 kDa band corresponding to the expected molecular mass of the p10-N fusion protein (data not shown).