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Development of an IFN-γ-recall assay for porcine reproductive and respiratory syndrome virus

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Meinen Eltern und meiner Schwester

"Imagination is more important than knowledge. Knowledge is limited. Imagination encircles the world".
(Albert Einstein)

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Abbreviations

α	Alpha
APC	Antigen presenting cell
Approx.	Approximately
Aqua dist.	Aqua destillata
β	Beta
BCIP	Bromochloroindolylphosphate
BHV	Bovine herpes virus
BSA	Bovine serum albumin
CO_2	Carbon dioxide
CPE	Cytopathic effect
Ct	Threshold cycle
DABCO	Diazabicyclooctan (=Triethylendiamin)
DMSO	Dimethyl sulfoxide
e.g.	Exempli gratia (for example)
ELISA	Enzyme-linked immunosorbent assay
EU	European
FCS	Fetal calf serum
FI	Field isolate
Fig.	Figure
FITC	Fluorescein isothiocyanate
γ	Gamma
g	Gram
GP	Glycoprotein
HP	High pathogen
HS	Horse serum
IF	Immunofluorescence
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
l	Litre
Li	Lithium
M	Mol

Mab	Monoclonal antibody
MAP	*Mycobacterium avium* ssp. *paratuberculosis*
MEM	Minimal Essential Medium
ml	Millilitre
µl	Microliter
MLV	Modified live virus
mM	Millimol
mOD	Milli OD
MOI	Multiplicity of infection
NA	North American
NBT	Nitro-blue tetrazolium
NC	Negative control
NEA	Non-essential amino acid
NGS	Normal goat serum
nm	Nanometres
NSP	Non-structural protein
OD	Optical density
ORF	Open reading frame
PAM	Porcine alveolar macrophages
PBS	Phosphate buffered solution
PC	Positive control
PCR	Polymerase chain reaction
PMBC	Peripheral mononuclear blood cells
Pen/Strep	Penicillin/Streptomycin
pg	Pico gram
p.i.	Post infection
PRRSV	Porcine Reproductive and Respiratory Syndrome Virus
p.v.	Post vaccination
PVDF	Polyvinylidenfluorid
RA	Recall assay
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute Medium
SC	Stimulation control
SNT	Serum neutralisation test

TCID	Tissue Culture Infection Dose
TMB	Tetramethylbenzidine
U	Units
US	United States
v/c	Vent/close
VN	Virus neutralising
w/o	Without
wt	Wild type

1. Introduction

Porcine Reproductive and Respiratory Syndrome (PRRS) is a virus induced infectious disease of pigs. It is also known as "Mystery Swine Disease" or "seuchenhafter Spätabort der Schweine". The virus belongs to the family of *Arteriviridae* from the order *Nidovirales* and is a small, enveloped positive-stranded RNA virus (Benfield et al., 1992; Conzelmann et al., 1993; Meulenberg et al., 1993; Zimmerman et al., 2012).

For the first time PRRS attracted attention in U.S. swine herds in the late 1980s and occurred in Europe in 1990 (Lindhaus and Lindhaus, 1991; Zimmerman et al., 2012). The causative agent was first isolated in the Netherlands in 1991 and designated *Lelystad virus* (Wensvoort et al., 1992b; Wensvoort et al., 1991).

Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) causes reproductive failure in sows leading to late-term abortions and is responsible for respiratory tract diseases in fattening pigs followed by a reduced average daily gain (Christianson et al., 1992; Lindhaus and Lindhaus, 1991; Terpstra et al., 1991; Zimmerman et al., 2012). Thus, it is a significant cause for economic losses (Holtkamp et al., 2013; Neumann et al., 2005). Complications by secondary bacterial infections (Drew, 2000; Zimmerman et al., 2012) lead to increased antibiotic treatments.

Vaccination is one of the most important measures to prevent PRRSV infection or its consequences (Alexopoulos et al., 2005; Mengeling et al., 1999; Zuckermann et al., 2007). Several commercial attenuated and inactivated vaccines are currently in use. However, efficacy of vaccination may be affected by the composition of the vaccine (Geldhof et al., 2012) or by genetic diversity among PRRSV field isolates (Labarque et al., 2004). Thanawongnuwech and Suradhat (2010) doubted effectiveness of currently available vaccines. Geldhof et al. (2012) mentioned the present demand for safe and more effective vaccines that induce protection against emerging virus variants. They assessed efficacy of different experimental and commercial PRRSV vaccines and gained deviating results for the vaccines regarding shortening of viremia.

The aim of several studies concerning PRRSV was and still is to find an acceptable possibility for prevention or at least control of PRRS. Molina et al. (2008) concluded

that current diagnostic tests are not able to determine the stage of PRRSV infection which is a big obstacle to the prevention and control of PRRS.

The Polymerase chain reaction (PCR) for the detection of virus genome and the detection of PRRSV-specific antibodies by an Enzyme-linked immunosorbent assay (ELISA) are regularly used in routine diagnosis after vaccination. However, these diagnostic tests do not control the success of vaccination. Antibodies detected with a commercial ELISA are not protective against PRRSV because they are not neutralising. This ELISA detects antibodies directed against the nucleoprotein. Neutralising antibodies are directed against epitopes on envelope glycoproteins (GP) of the virus (Cancel-Tirado et al., 2004). Furthermore, except for the detection of vaccine virus, positive PCR results in vaccinated stocks indicate the failure of vaccination or the infection with a heterologous virus. Therefore, these diagnostic tests (PCR and ELISA) do not predict the success of vaccination.

An indicator for success of vaccination is the formation of a protective immune response. By measuring parameters of a protective immune response, i.e. neutralising antibodies or interferon-(IFN-)-γ, vaccine management might be optimised and failure of vaccination might be avoided.

The present study was created to establish a simple, robust and cost-effective IFN-γ-recall assay (RA) for PRRSV diagnostics. The objective of this study was to assess IFN-γ-response in order to predict immune protection after vaccination. Thus, it should be possible, with the aid of SNT and IFN-γ-RA, to assess PRRSV herd immunity and recognise gaps in immunity earlier.

2. Literature

PRRSV does not represent a uniform virus. Several strains of PRRSV exist. Not only the European (EU, genotype 1) and North American (US, genotype 2) PRRSV isolates are different to each other (Albina, 1997; Nelson et al., 1993; Wensvoort et al., 1992a). Also within the EU- or US-genotypes genetic and antigenic differences exist (Forsberg et al., 2002; Kapur et al., 1996; Oleksiewicz et al., 2000; Wensvoort et al., 1992a).

These genetic and antigenic differences affect immune response against PRRSV. Infection results in a protective immune response against a re-infection with the homologous virus strain (Ohlinger et al., 1991). It develops within 60 days post infection (p.i.) and lasts for a minimum of 600 days (Lager et al., 1999, Lager et al., 1997a, Lager et al., 1997b). Contrary, a protective immunity against a heterologous virus strain is incomplete (Labarque et al., 2004; Lager et al., 1999; Mengeling et al., 1999). Furthermore, it does not last as long as homologous protection (Lager et al., 1999). On the basis of a cell-mediated immune response, Zuckermann et al. (2007) found that modified live virus (MLV) vaccines established a protective immunity against a challenge with a PRRSV strain that has a homology of 93% to the vaccine virus. In contrast, a killed vaccine virus did not protect against a challenge virus strain with 99% homology to the vaccine virus.

PRRSV proteins are encoded by eight open reading frames (ORF's), ORF1a and 1b encoding the RNA polymerase, ORF 2-4 encoding the minor membrane associated proteins and ORF5, 6 and 7 encoding GP5, the non-glycosylated membrane protein and the non-glycosylated nucleocapsid protein (Meulenberg et al., 1993; Meulenberg et al., 1995). GP5 and the nucleoprotein as well as the non-glycosylated membrane protein belong to the major structural and immunogenic proteins of PRRSV.

Both humoral and cellular immunity are important for PRRSV immunity. The development of ELISA antibodies and neutralising antibodies as well as IFN-γ-producing cells over time was described by Lopez and Osorio (2004).

Infection with virulent PRRSV or immunisation with a PRRS-MLV-vaccine induces an early increase of non-neutralising antibodies within two weeks (Díaz et al., 2005; Nelson et al., 1994; Yoon et al., 1995). Such ELISA antibodies reach their maximum within one to two months and begin to decline after three months (Molitor et al., 1997; Yoon et al., 1995). Non-neutralising antibodies are directed against the nucleocapsid

protein (Cancel-Tirado et al., 2004) and against one of two epitopes on the PRRSV GP5: epitope A, an immunodominant, non-neutralising determinant (Gonin et al., 1999; Ostrowski et al., 2002). Epitope B, a neutralising determinant, is a target for VN antibodies (Gonin et al., 1999; Ostrowski et al., 2002).

Virus neutralising (VN) antibodies appear coincidently with the decline of non-neutralising antibodies (Meier et al., 2003; Shibata et al., 2000). VN antibodies are detected within four to eight weeks by Molitor et al. (1997) and Díaz et al. (2005) with the highest level at 12 weeks p.i. Osorio et al. (2002) found that VN antibodies are protective against subsequent PRRSV infections. Passive transfer of VN antibodies protects against an infection with a homologous virus strain (Osorio et al., 2002). Glycosylation of GP5, as it can be found in wild type (wt) PRRSV, inhibits the induction of VN antibodies because glycans mask the neutralising epitope on GP5 (Ansari et al., 2006). Current PRRSV isolates showed additional glycosylation sites compared to early virus strains (Pesch et al., 2005). In vaccines the removal of glycans can improve VN antibodies not only against the homologous vaccine virus but also against wt PRRSV (Ansari et al., 2006). Böttcher et al. (accepted) assessed the level of neutralising antibodies against the EU- and US-vaccine virus and compared it with detection of PRRSV by PCR in the group of weaned piglets. The highest level of EU-SNT-titers was observed in EU-vaccinated sows without concurrent circulation of PRRSV-EU in weaned piglets. In contrast, detection of PRRSV-EU coincided with a significantly lower level of EU-SNT-titers in sows. Moreover, sows of 1./2. parity had significantly lower SNT-titers compared to sows of higher parity in these herds.

Virus-specific IFN-γ-secreting cells appear from day 14 onwards, plateauing at six months post infection (p.i.) or post vaccination (p.v.) (Díaz et al., 2005; Meier et al., 2003; Ohlinger et al., 1991). Porcine IFN-γ blocks PRRSV replication in macrophages by inhibiting the viral RNA synthesis (Bautista and Molitor, 1999; Rowland et al., 2001).

A correlation between protection and IFN-γ-secreting cells was observed by Lowe et al. (2005) for infection and by Zuckermann et al. (2007) and Martelli et al. (2009) after vaccination. But Meier et al. (2003) hypothesised that differentiation of virus-specific T-cells into virus-specific IFN-γ-secreting cells is not adequate after exposure to virulent or attenuated PRRS virus.

IFN-α (or type I IFN), a cytokine secreted by dendritic cells after virus infection, stimulates the differentiation of T-cells into IFN-γ-secreting cells (Cella et al., 2000; Kadowaki et al., 2000). Meier et al. (2004) described a three-fold increase of the PRRSV specific IFN-γ-response when IFN-α was co-administered at vaccination, but PRRSV is able to escape the immune response. Five non-structural proteins (NSP), NSP1α, NSP1β, NSP2, NSP4 and NSP11, inhibit IFN-α production (Beura et al., 2010; Royaee et al., 2004). As a consequence, IFN-γ-secreting cells are reduced. Nevertheless, even at low level, IFN-γ might promote the differentiation of naive T-cells into virus-specific IFN-γ-secreting cells leading to a gradual increase in the IFN-γ-response (Meier et al., 2003). Contrary, Sipos et al. (2003) did not detect changes in IFN-γ after the pigs were exposed to PRRSV. This investigation may be an indicator for the variability of IFN-γ-responses of pigs against PRRSV.

Moreover, PRRSV isolates induce secretion of Interleukin-6 (IL-6) and IL-10 early after infection (Royaee et al., 2004; Zuckermann et al., 2007). IL-6 produced by antigen presenting cells (APC) was found to promote the differentiation of activated B-cells into plasma cells secreting antibodies on the one hand and to inhibit IFN-γ-production on the other hand (Diehl and Rincón, 2002). This situation (type I IFN↓, IL-6↑, IL-10↑) might favour the formation of antibodies (Royaee et al., 2004) and might delay the cellular immune response (Meier et al., 2003). Charerntantanakul et al. (2006) reported reduced IFN-γ-expression in T-cells cultured with virulent PRRSV-infected monocytes. This suppressive activity seems to be associated with virulence as it was absent after exposure to attenuated vaccine virus (Charerntantanakul et al., 2006). As GP5 of PRRSV was found to be a relevant factor for activating humoral immunity, it was also examined for its ability to stimulate IFN-γ-secreting cells.

Using the IFN-γ-ELISpot assay, Vashisht et al. (2008) identified two T-cell epitopes on GP5 of an US-field strain stimulating IFN-γ-production in PBMC. Díaz et al. (2009) found T-cell epitopes not only in GP5 of an EU-PRRSV, but also additional immunodominant epitopes on nucleocapsid and GP4. Nucleocapsid and GP4 also seem to be more immunodominant than GP5 (Díaz et al., 2009). Wang et al. (2011) finally investigated the membrane protein of PRRSV for immunodominant epitopes and identified T-cell epitopes on some peptides of highly virulent PRRSV isolates.

The importance of IFN-γ in the course of PRRSV infection became obvious regarding the study of Díaz et al. (2005), who found that the last detection of viremia in infected pigs corresponded to the appearance of IFN-γ-secreting cells.

VN antibodies and IFN-γ-response after *in vitro* stimulation of PBMC with viral antigens are suitable prognostic parameters of immunity for diagnostic tests. How VN antibodies or IFN-γ-responses, e.g. after vaccination, can be used to predict protection against infection has not been determined yet (Lowe et al., 2005; Lowe et al., 2006; Molina et al., 2008; Zuckermann et al., 2007).

Regarding non-neutralising and VN antibodies as well as IFN-γ-response, vaccination policy needs to be combined with monitoring the efficiency of vaccination. The assessment of the IFN-γ-response either by IFN-γ-ELISpot or IFN-γ-RA and the use of SNT might be appropriate in order to predict protection against and to prevent an infection with PRRSV.

3. Material and Methods

Information about used materials is given in annex 1.

3.1. Preliminary work

3.1.1. Cell culture

MARC145 cells were used for propagation of vaccine virus. Cells were cultured in MEM Earle's (BIOCHROM AG) supplemented with 10 mM HEPES-Buffer, 1% NEA and 1% Pen/Strep (EMEM). Porcine Alveolar Macrophages (PAM) were used for propagation of field virus strains. Macrophages were cultured in RPMI 1640 (BIOCHROM AG) supplemented with 1% NEA, 1% Pen/Strep and 1% L-glutamine (RPMI).

3.1.1.1. MARC145

MARC145 cells (Kim et al., 1993) were kindly provided by the Friedrich-Loeffler-Institute, Riems. The cells were passaged in cell culture vessels with EMEM/FCS10% and incubated at 37°C without CO_2. Confluent monolayers were inoculated with vaccine derived PRRSV. For virus titration and serum neutralisation MARC145 cells were seeded on a 96-well microtiter plate at a density of 50.000 cells/well. The plate was incubated at 37°C and 5% CO_2. Two days later, when the cell monolayer was confluent, the cells were used for further tests. All in all, the cells were used for up to 30 passages for virus propagation.

3.1.1.2. Porcine Alveolar Macrophages (PAM)

PAM (Mengeling et al., 1995; Wensvoort et al., 1991) were harvested from lungs of piglets as previously described (Mengeling et al., 1995; Wensvoort et al., 1991; Yoon et al., 1992), with some minor modifications of the procedure. Piglets with approx. 20 kg were obtained from a stock tested free of PRRSV by antibody ELISA, SNT and PCR. They tested negative (PCR) for porcine circovirus type 2, swine influenza virus and *Mycoplasma hyopneumoniae*. Lungs were flushed five to ten times with cold

(4°C) RPMI 1640 supplemented with 1% Pen/Strep, 1% patricin and 0.04% baytril 5%. Lavage fluid was filtered through gauze and collected in a sterile vessel of glass on ice. Lavage fluid was sedimented at 750 x g for 10 minutes. Pellets of PAM were resuspended in 10 ml RPMI/FCS10% for cell counting. Then PAM were sedimented once more and resuspended in medium containing 40% RPMI 1640 (including 1% NEA and 1% L-glutamine), 50% FCS and 10% DMSO for storage in liquid nitrogen. The cell number for freezing was set to a concentration of 1.2×10^6 cells/ml. Aliquots of 1 ml were stored in liquid nitrogen. PAM were examined for sterility on blood agar as well as for PRRSV, porcine circovirus type 2, swine influenza virus and *Mycoplasma hyopneumoniae* by PCR.

For use, PAM were thawed, sedimented by centrifugation and resuspended in RPMI/HS10% at a density of 10^5 cells/ml. After overnight incubation at 37°C and 5% CO_2, PAM were used for propagation of field virus.

3.1.1.3. Counting of cells

Cell suspension was appropriately diluted with PBS and trypan blue (890 µl PBS, 100 µl trypan blue, 10 µl cell suspension). A Fuchs-Rosenthal counting chamber was used for counting cells and cell numbers per ml were calculated in consideration of dimension of the counting chamber and the pre-dilution.

3.1.2. Titration of virus

Virus titers were determined according to Wills et al. (1997) on MARC145 cells for vaccine virus and on PAM in case of field virus strains in 96-well microtiter plates. For titration, ten log10-dilutions of virus were prepared. In case of vaccine derived antigens, 50 µl/well of each virus dilution were inoculated on a monolayer of MARC145 cells in six replicates and incubated for 1 hour at 37°C and 5% CO_2. Then 50 µl/well EMEM/FCS10% were added. In case of field virus derived antigens, 10 µl of field virus strain dilutions were given in six replicates to 90 µl of PAM in RPMI/FCS10% (approx. 10.000 cells/well). Six more wells were filled with medium as cell control. After 5-7 days, wells of each microtiter plate were examined for cytopathic effect (CPE). The titer of virus was determined according to the method of Kaerber (Schmidt & Emmons, 1989):

$$\text{Negative logarithm of TCID}_{50} = x_0 - (((\Sigma\%CPE/100) - 0.5) \times (\log d))$$

x_0 = logarithm of the highest virus concentration used

$\Sigma\%CPE$ = sum of % cytopathic effect at each dilution

$\log d$ = logarithm of dilution series (1 in the case of log10-dilution)

3.1.3. Propagation of virus

Vaccine virus was re-isolated from two commercially available vaccines (Ingelvac® PRRS MLV, Boehringer, Ingelheim; genotype 2; US vaccine virus strain and Porcilis® PRRS, MSD/Intervet, Boxmeer; genotype 1; EU vaccine virus strain) on MARC145 cells. Virus was passaged for 12 times. Four field virus strains FI I-IV (V2276/I/2012, V1192/2013, V683/2013, V995/2013) were isolated from tissues from pigs. Lungs, spleens and afterbirths were homogenized and inoculated to PAM at a ratio of one to ten. Cells were observed daily for CPE for up to 7 days after inoculation. When CPE was observed, supernatant was used for further passages of the virus in PAM (five to six passages).

The titer and number of passage for seed virus stocks are summarized in Table 1.

Sequencing of the isolated field virus strains was done by IVD GmbH (Gesellschaft für Innovative Veterinärdiagnostik mbH, Hannover). Based on the ORF5 gene the isolates FI I, FI II and FI III were compared with the reference strain Lelystad virus. The isolate FI IV was compared with the reference strain Lelystad virus by ORF7 because amplification of ORF5 was not successful for this isolate. The segment of ORF7 is shorter than the segment of ORF5 (Fig. 1). The PRRSV nucleotide sequences of FI I, FI II, FI III and FI IV shared a homology of 89%, 89%, 87% and 93% to the PRRSV EU reference strain Lelystad in ORF5 (FI I, FI II, FI III) and ORF7 (FI IV) respectively.

Table 1: Characteristics of seed virus stocks.

	passage	titer
vaccine virus		
US	12th	$10^{7.5}$/ml
EU	12th	$10^{7.8}$/ml
field virus		
FI I	6th	$10^{4.13}$/ml
FI II	5th	$10^{5.1}$/ml
FI III	5th	10^{6}/ml
FI IV	5th	$10^{5.8}$/ml

Source: Own presentation

3.1.3.1. Antigen preparations – vaccine derived virus

The value of different PRRSV antigen preparations had to be assessed for IFN-γ-stimulation. Two vaccine derived virus strains were used. Three antigen preparations of US and EU vaccine virus were produced as cell culture supernatants: antigen in EMEM/FCS10%, antigen in EMEM w/o FCS and cell-lysates of infected cells in PBS. MARC145 cells were rinsed with PBS and inoculated with virus in EMEM w/o FCS. **Antigen in EMEM/FCS10%:** cell culture (30th passage) was inoculated with a multiplicity of infection (MOI) of 0.1. After incubation for 1 hour at 37°C and 5% CO_2, the culture vessel was half filled with EMEM/FCS10%. Cell cultures were incubated 48 hours (CPE ~ 75%) at 37°C and 5% CO_2.

An uninfected MARC145 cell culture was prepared as control antigen (MARC145/FCS). Cells were rinsed with PBS, covered with EMEM w/o FCS for 1 hour at 37°C and 5% CO_2 and then half refilled with EMEM/FCS10%. Uninfected cell culture was incubated 48 hours at 37°C and 5% CO_2.

Antigen in EMEM w/o FCS: cell culture (31th passage) was inoculated with a MOI of 1. After incubation for 1 hour at 37°C and 5% CO_2, the culture vessel was half filled with EMEM/FCS10%. Six hours later, medium was discarded, cells were rinsed with PBS and covered again with EMEM w/o FCS. Cell cultures were incubated additional 40 hours (CPE ~ 25%) at 37°C and 5% CO_2.

An uninfected MARC145 cell culture was prepared as control antigen (MARC145 w/o FCS). Cells were rinsed with PBS, covered with EMEM w/o FCS for 1 hour at 37°C

and 5% CO_2 and then half refilled with EMEM/FCS10%. Six hours later, medium was discarded, cells were rinsed with PBS and covered again with EMEM w/o FCS. Uninfected cell culture was incubated additional 40 hours at 37°C and 5% CO_2.

Cellysate-antigen in PBS: cell culture (29th passage) was inoculated with a MOI of 1. After incubation for 1 hour at 37°C and 5% CO_2, the culture vessel was half filled with EMEM/FCS10%. Sixteen hours later (beginning CPE), supernatant was discarded and the cells were collected in a tenth volume of PBS.

An uninfected MARC145 cell culture was prepared as control antigen (MARC145). Cells were rinsed with PBS, covered with EMEM w/o FCS for 1 hour at 37°C and 5% CO_2 and then half refilled with EMEM/FCS10%. Sixteen hours later, supernatant was discarded and cells were collected in a tenth volume of PBS.

Next, the cells of the different antigen preparations were lysed by three freeze-thaw cycles (-20°C/25°C). Lysate was cleared by centrifugation at 250 x g for 10 minutes. Supernatant was aliquoted and stored at -80°C. Antigens and virus titers are summarized in Table 2.

3.1.3.2. Antigen preparations – field virus strains

Isolates FI I-IV were used as seed virus. PAM cultures were infected with a MOI of 0.01. The cell culture medium contained 10% horse serum. After three days, CPE was 100%. The remaining cell particles were sedimented by centrifugation at 250 x g for 10 minutes. The supernatants were aliquoted and stored at -80°C. The CPE was confirmed by indirect immunofluorescence. The field virus antigens used for stimulation had titers of 10^5/ml (FI I), $10^{4.2}$/ml (FI III) and 10^4/ml (FI II, FI IV).

An uninfected PAM cell culture was prepared as control antigen (PAM). The cell culture was incubated in RPMI for three days. After one freeze-thaw cycle, remaining cell particles were sedimented by centrifugation at 250 x g for 10 minutes. The supernatants were aliquoted and stored at -80°C.

3.1.4. Storage of antigens

Aliquots of antigens and control antigens were stored at -80°C and for examination of stability at -20°C and +2 - 8°C. The stimulation control was stored at -20°C.

```
              10        20        30        40        50        60        70        80        90       100
     ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
0 ATGAGATGTTCTCACAAATTGGGGCGTTTCTTGACTCCGCACTCTTGCTTCTGGTGGCTTTTTTTGCTGTGTACCGGCTTGTCCTGGTCCTTTGCCGATG
1 ...............................T...................T.....................T...........T..............
2 ....A.........................G...............A...T...............................C.................
3 ........G..................T.....C.................C..CCT...................T.......................

             110       120       130       140       150       160       170       180       190       200
     ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
0 GCAACGGCGACAGCTCGACATACCAATACATATATAACTTGACGATATGCGAGCTGAATGGGACCGACTGGTTGTCCAGCCATTTTGGTTGGGCAGTCGA
1 ........A..................G..C.....................AC.........A..G.T..C..TA.....T......
2 ...A.................................A.....................T.C.CA....CA.G.T..C...AA....T......
3 .................TC..........................C.......CT.......T.A...C....A.........

             210       220       230       240       250       260       270       280       290       300
     ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
0 GACCTTTGTGCTTTACCCGGTTGCCACTCATATCCTCTCACTGGGTTTTCTCACAACAAGCCATTTTTTTGACGCGCTCGGTCTCGGCGCTGTATCCACT
1 .......................A..C..C..T......T.............G.......................T..............G...GTC
2 .......................A....C..T..............T..............T..AG..........G...GTC
3 ........T........................A......T.......................C...A.......G....TC

             310       320       330       340       350       360       370       380       390       400
     ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
0 GCAGGATTTGTTGGCGGGCGGTACGTACTCTGCAGCGTCTACGGCGCTTGTGCTTTCGCAGCGTTCGTATGTTTTGTCATCCGTGCTGCTAAAAATTGCA
1 A....G...TA..A......A...AC....A...TA.T...........C.........C....G.........T................
2 .....G...TG..A.AA...A..T......A...TA.T........T........C....C...C.....G..C....C...T....T..........
3 A....G...TA.A.AA...A........A...TA.........C..........C.........C....C.........C.........T.

             410       420       430       440       450       460       470       480       490       500
     ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
0 TGGCCTGCCGCTATGCCCGTACCCGGTTTACCAACTTCATTGTGGACGACCGGGGGAGAGTTCATCGATGGAAGTCTCCAATAGTGGTAGAAAAATTGGG
1 ....T.........C....C.........C..........T..C...........GA.......G....G...........G..G...C....
2 ....T.........C....C.........C................A....AGA.C...G....G...........G..G...C....
3 ....T.......T....C..T.................GA...............G..G...CA...

             510       520       530       540       550       560       570       580       590       600
     ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
0 CAAAGCCGAAGTCGATGGCAACCTCGTCACCATCAAACATGTCGTCCTCGAAGGGGTTAAAGCTCAACCCTTGACGAGGACTTCGGCTGAGCAATGGGAG
1 ......T...A.T.GC.........T.......................................C.A...............
2 T.....T...A.T.GC...GC...T..TG..........................T.......C................
3 ...G......A.T.GC.T.G.......C...........A..........G........G..........A........C...........

     ....|.
0 GCCTAG
1 ......
2 ......
3 ......
```

a

```
              10        20        30        40        50        60        70        80        90       100
     ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
0 ATGGCCGGTAAAAACCAGAGCCAGAAGAAAAAGAAAAGTACAGCTCCGATGGGGAATGGCCAGCCAGTCAATCAACTGTGCCAGTTGCTGGGTGCAATGA
4 ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

             110       120       130       140       150       160       170       180       190       200
     ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
0 TAAAGTCCCAGCGCCAGCAACCTAGGGGAGGACAGGCCAAAAAGAAAAAGCCTGAGAAGCCACATTTTCCCCTGGCTGCTGAAGATGACATCCGGCACCA
4 ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~....C...T...........................T..

             210       220       230       240       250       260       270       280       290       300
     ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
0 CCTCACCCAGACTGAACGCTCCCTCTGCTTGCAATCGATCCAGACGGCTTTCAATCAAGGCGCAGGAACTGCGTCGCTTTCATCCAGCGGGAAGGTCAGT
4 .........A.T......T.......T.............C..T..C.........G...........

             310       320       330       340       350       360       370       380
     ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|..
0 TTTCAGGTTGAGTTTATGCTGCCGGTTGCTCATACAGTGCGCCTGATTCGCGTGACTTCTACATCCGCCAGTCAGGGTGCAAGTTAA
4 ........C.....C.....................
```

b

Figure 1: Genetic analysis of field isolates – sequence data

0: reference strain Lelystad, 1: FI I, 2: FI III, 3: FI II, 4: FI IV; a/b: Sequencing of ORF5 (a) or ORF7 (b).

Source: IVD GmbH

Table 2: Antigens and control antigens

	titer	control antigen
US in EMEM/FCS	$10^{7.5}$/ml	MARC145/FCS
EU in EMEM/FCS	$10^{7.8}$/ml	MARC145/FCS
US in EMEM w/o FCS	$10^{6.3}$/ml	MARC145 w/o FCS
EU in EMEM w/o FCS	$10^{6.1}$/ml	MARC145 w/o FCS
US in PBS	$10^{8.6}$/ml	MARC145
EU in PBS	$10^{8.3}$/ml	MARC145
FI I	10^{5}/ml	PAM
FI II	10^{4}/ml	PAM
FI III	$10^{4.2}$/ml	PAM
FI IV	10^{4}/ml	PAM

Source: Own presentation

3.2. Detection of antibodies and antigens

3.2.1. Antibody ELISA

A commercial enzyme linked immunosorbent assay (indirect ELISA) was used to detect antibodies against PRRSV in serum samples (Herd Chek* PRRS X3, Porcine Reproductive and Respiratory Syndrome Virus Antibody Test Kit, IDEXX Laboratories). The IDEXX ELISA was performed following the kit instructions. All reagents were provided. Diluted serum samples and undiluted positive (PC) and negative controls (NC) were added to the wells of the test plate. Test plates were coated with recombinant PRRSV antigen (PRRSV capsid protein). Antibodies specific for PRRSV bound to the coated antigens during an incubation period of 30 minutes at room temperature. Unbound material was removed by washing the plate five times. An anti-porcine immunoglobulin linked to horseradish peroxidase was added, binding to the porcine antibody fixed in the wells. After incubation for 30 minutes at room temperature and washing the plate five times, TMB substrate was added and incubated for 15 minutes at room temperature. The stop solution finished the enzymatic reaction.

Optical density (OD) was measured at 650 nm with a microplate reader for ELISA assays (Sunrise™, Tecan Austria GmbH, Grödig). Reactivity of samples was calculated with the following formula:

$$\% \ reactivity_{sample} = ((OD_{sample} - OD_{NC}) / (OD_{PC} - OD_{NC})) * 100$$

As the positive and negative controls were tested in duplicate, the average value of the two measured values for each control was used for calculation.

Evaluation considered a threshold area of 30-40% reactivity.

3.2.2. Serum neutralisation test (SNT)

Titers of VN antibodies were determined by the SNT according to Yoon et al. (1994) and modified by Böttcher et al. (2006) against EU and US vaccine virus. A heat-treatment of sera (water bath, 60°C, 30 minutes) was performed. Vaccine virus was diluted to 200 $TCID_{50}$/50µl in EMEM supplemented with 4% serum from guinea pigs as a source of complement. Heat-inactivated sera were diluted 1/2, 1/4 and 1/8 in EMEM in an empty 96-well microtiter plate with a final volume of 50 µl/well. 50 µl Virus (200 $TCID_{50}$/50µl) were added. Wells with medium only, a back-titration of infectivity and a 1/2-dilution of sera without virus were included as additional controls. The plate was incubated for 1 hour at 37°C and 5% CO_2. The supernatant of a microtiter plate with confluent cells was discarded and 50 µl from each well of the pre-incubation plate were transferred. After another incubation (1 hour at 37°C and 5% CO_2), 50 µl/well EMEM/FCS10% were added. The test was incubated for 5-6 days at 37°C and 5% CO_2. Cells were regularly examined for CPE. The serum control was checked for cytotoxicity.

The neutralisation titer of antibodies was calculated by the method of Kaerber (Schmidt & Emmons, 1989) and expressed as <2; 2.8; 4; 5.6; 8 and ≥11.2.

3.2.3. Real-time reverse transcriptase polymerase chain reaction (PCR)

PCR was performed to detect and quantify viral RNA in serum. RNA was extracted from serum samples with the QIAamp® Viral RNA Mini-Kit (QIAGEN®) following the kit instructions. The samples were mixed by pulse-vortexing in a 1.5 ml micro tube. The micro tube contained a prepared Buffer AVL-carrier RNA (provided by QIAGEN®) and serum. Incubation at room temperature led to viral particle lysis. Ethanol was added to the sample. The solution was applied to a QIAamp Mini spin column in a 2 ml collection tube (QIAGEN®). By centrifugation, RNA bound to the QIAamp membrane. Contaminants were washed away in two steps using two different wash buffers (Buffer AW1 by QIAGEN® and Buffer AW2 by QIAGEN®). RNA was eluted in a 1.5 ml microcentrifuge tube with RNAse-free buffer (Buffer AVE by QIAGEN®). RNA could be used directly for PCR. PCR was carried out with VIROTYPE® PRRSV (Real-time Multiplex RT-PCR Test Kit for Detection of EU, NA and HP PRRS Viruses, Labor Diagnostik GmbH Leipzig) including enzymes, primers

and probes in one mixture (PRRSV-Mix) as well as a positive and a negative control. The test kit can be used to detect EU- and US-genotype of PRRSV, a highly pathogenic (HP) strain of US-genotype and an amplification- and extraction control (mRNA of β-actin housekeeping gene) at the same time. PRRSV-Mix was prepared with the RNA elution using Optical Tube Strips (Agilent Technologies). The formulation per sample included 80% of PRRSV-Mix and 20% of sample or controls. PCR was performed on Stratagene M3005P (Agilent Technologies). The profile of temperature and time is shown in table 3.

Analysis was based on threshold cycle (Ct) determinations using the Mx Pro QPCR Software (Agilent Technologies).

Table 3: Temperature profile for PCR

		time period	temperature
reverse transcriptase		10 min	45°C
taq polymerase		10 min	95°C
	Denaturation	15 sec	95°C
	Annealing	30 sec	55°C
40 series	Measuring		
	Elongation	30 sec	72°C

Source: VIROTYPE® PRRSV, Instructions for Use, Labor Diagnostik GmbH Leipzig

3.2.4. Indirect immunofluorescence

Indirect immunofluorescence (IF) was used to confirm virus induced CPE in cell cultures. Cells were fixed with acetone on a slide and incubated with an anti-PRRSV antibody (BioX Diagnostics, diluted 1:20) for 1 hour in a humid chamber at 37°C. Cells were washed three times with PBS (pH 7.4) for 5 minutes. An Fluorescein isothiocyanate (FITC) conjugated anti-mouse IgG (Sigma-Aldrich®, diluted 1:250) was added. After another incubation period for 1 hour in a humid chamber at 37°C, cells were washed again as described above and were subsequently covered with DABCO buffer (PBS and glycerol in equal volumes supplemented with 3.5% DABCO) and a coverglass. Cells were examined with a fluorescence microscope (20x magnification, Intensilight C-HGFI, Nikon). An uninfected cell culture also was prepared for indirect immunofluorescence as negative control.

3.3. Detection of IFN-γ

3.3.1. Counting of peripheral blood mononuclear cells (PBMC)

Peripheral blood mononuclear cells (PBMC) were counted (Cell-Dyn 3500, Abbott, Illinois). Automated counting was initially confirmed by a Neubauer counting chamber.

3.3.2. Stimulation of blood samples with antigens

Stimulation of PBMC in Li-Heparin-stabilized blood was principally performed according to Böttcher et al. (2010). Blood samples were stimulated within 8 hours after collection. Stimulation was performed in a laminar flow with sterile equipment. Viral antigens (US, EU) and control antigen (MARC145) were heat-inactivated in a water bath (60°C, 30 minutes). Pokeweed mitogen (Sigma-Aldrich®), activating humoral and cellular immune response (Mellstedt, 1975), was used as a stimulation control (SC) and thus as a positive control for functionality of PBMC. PBS served as a further negative control. US, EU, MARC145 and SC were prediluted to a working concentration in PBS. Diluted antigens, control antigen, SC and PBS (each 20 µl) were distributed to appropriate wells of a cell-culture microtiter plate. Blood samples were thoroughly mixed end-over-end and 280 µl whole blood were distributed to appropriate wells with antigens and controls. The microplate was shaked to mix antigen and blood. Each blood sample was stimulated in duplicate. Stimulation was performed for 16 hours at 37°C and 5% CO_2. The next day, plasma was separated by centrifugation at 500 x g for 5 minutes and transferred to a storage microtiter plate. Plasma was either immediately transferred to the IFN-γ-RA test plate or stored at -20°C for later use.

The following variations of the basic protocol were included:

Additional controls: control antigen prepared in PBS (MARC145/PBS), EMEM w/o FCS and PBS/FCS10% were prepared for stimulation of blood samples in order to assess any reactivity against medium components.

FCS and horse serum (HS): PBS/FCS10%, PBS/FCS1%, PBS/HS10% and PBS/HS1% were prepared for stimulation of blood samples in order to assess any reactivity against serum components.

Field virus and PAM: four field isolates FI I-IV and the PAM control were included for stimulation of blood samples.

3.3.3. IFN-γ-recall assay (IFN-γ-RA)

Two porcine IFN-γ kits, test kit A (R&D Systems®) and B (Mabtech), were compared regarding user-friendliness, detection limit and costs. Annex 2.1, annex 2.2 and annex 3 are summarising the components of the used test kits and their implementation.

Test kit A: a high protein binding ELISA plate (Nunc-Immuno™ Plates, MaxiSorp) had to be coated with a monoclonal antibody specific for porcine IFN-γ (mouse anti-porcine IFN-γ). Mab was diluted in PBS (pH 7.4) to 2 µg/ml. Coating was performed overnight at 4°C. The antibody coated microtiter plate was washed three times with wash buffer and blocked with Reagent Diluent for 1 hour at room temperature. After another three washing procedures the plate was ready for use.

Upon arrival, the standard was reconstituted in Reagent Diluent to a concentration of 0.075 µg/ml and stored in aliquots at -20°C until use. Using 2-fold serial dilutions, a seven point standard curve with IFN-γ-concentrations ranging from 62.5 to 4000 pg/ml was performed. Reagent Diluent served as zero negative value.

Stimulation of blood samples with antigens should result in IFN-γ-production of PBMC if PRRSV infection or vaccination had been taken place before. Plasma samples were diluted 1:2 in Reagent Diluent. The samples and the undiluted standard series were transferred to the test plate. IFN-γ bound to the immobilized antibody on the microtiter plate within an incubation period of 2 hours at room temperature. The biotinylated polyclonal antibody, diluted in Reagent Diluent with 2% heat inactivated normal goat serum (NGS) to 0.4 µg/ml (incubation period 2 hours), and Streptavidin-Horseradish Peroxidase, diluted in Reagent Diluent 1:200 (incubation period 20 minutes), were added. Every incubation period was followed by three washing steps to remove unbound material. Tetramethylbenzidine (CHECKIT* TMB substrate, Idexx Laboratories) was used as substrate solution and enzymatic reaction was stopped after 20 minutes with an appropriate stop solution (CHECKIT* stop solution TMB, Idexx Laboratories).

Test kit B: a high protein binding ELISA plate (Nunc-Immuno™ Plates, MaxiSorp) had to be coated with a monoclonal antibody specific for porcine IFN-γ (mouse anti-

porcine IFN-γ). Mab was diluted in PBS (pH 7.4) to 2 µg/ml. Coating was performed overnight at 4°C. The antibody coated microtiter plate was washed twice with PBS and blocked with the incubation buffer for 1 hour at room temperature. After another five washing procedures the plate was ready for use.

Upon arrival, the standard was reconstituted in PBS with 0.1% BSA to a concentration of 0.5 µg/ml and stored in aliquots at -20°C until use. 10, 100 and 1000 pg/ml and incubation buffer (zero value) served as standard curve.

Plasma samples were diluted 1:2 in incubation buffer. The samples and the undiluted standard series were transferred to the test plate. IFN-γ bound to the immobilized antibody on the microtiter plate within an incubation period of 2 hours at room temperature. The biotinylated monoclonal antibody, diluted in incubation buffer to 0.5 µg/ml (incubation period 1 hour), and Streptavidin-Horseradish Peroxidase, diluted in incubation buffer 1:1000 (incubation period 1 hour), were added. Every incubation period was followed by five washing steps to remove unbound material. TMB substrate (CHECKIT* TMB substrate, Idexx Laboratories) was used as substrate solution and enzymatic reaction was stopped after 10 minutes with an appropriate stop solution (CHECKIT* stop solution TMB, Idexx Laboratories).

Measurement: OD's were measured at 450 nm with a photometer for ELISA assays (Sunrise™, Tecan Austria GmbH).

Calculation: plasma samples were tested in duplicates. The two values (OD) were averaged. Reactivity to PBS control was subtracted from that to SC ($OD_{SC}-OD_{PBS}$) and control antigen ($OD_{MARC145}-OD_{PBS}$). Thus, specific reactivity to SC and MARC145 could be examined. PBS control and the control antigen were used as negative controls. Reactions occurring in these controls had to be differentiated from specific reactivity to US- and EU-antigen. To get the specific reactivity to these two antigens, OD of MARC145 (before subtracting PBS) was subtracted from OD to US- and EU-antigens ($OD_{us}-OD_{MARC145}$ and $OD_{EU}-OD_{MARC145}$) respectively. Specific (US, EU) and control antigens (PBS, MARC145) were expressed as a percentage of ($OD_{SC}-OD_{PBS}$).

The standards were tested in duplicate, which were averaged and corrected by subtraction of zero value (standard diluent). The standard curve was controlled for regularity. If needed, ODs of the standard curve were used to calculate the amount of IFN-γ produced by PBMC of the samples.

3.3.4. IFN-γ-ELISpot

An IFN-γ-ELISpot (ELISpot for Porcine IFN-γ, R&D Systems) was performed according to the kit instructions. All reagents were provided with the test kit.

Purification of PBMC: using Ficoll-Paque™ PLUS (GE Healthcare), PBMC were separated according to the manufacturer's instructions. Li-Heparin-stabilized blood samples were mixed with PBS in equal volumes. Four ml of diluted blood sample were carefully layered on 3 ml Ficoll-Paque™ PLUS. Centrifugation at 400 x g for 30 minutes at 20°C led to separation of blood cells. The PBMC layer was separated and suspended in PBS. Two additional centrifugation steps at 100 x g for 10 minutes each removed remaining platelets, Ficoll-Paque™ PLUS and plasma. PBMC then were resuspended in RPMI and used for stimulation.

Preparation of the ELISpot plate: the PVDF-backed microplate coated with a Mab specific for porcine IFN-γ was covered with RPMI/HS10%, and incubated for 20 minutes at room temperature. The culture medium was aspirated and the plate was ready for immediately use.

Stimulation: cell suspensions including the stimulatory agents were transferred to the test plate. 2.5×10^5 PBMC/well were stimulated with the same antigens and dilutions used for the IFN-γ-RA. The plate was incubated at 37°C and 5% CO_2 for 20 to 24 hours.

IFN-γ-ELISpot: cell suspension was discarded and the plate was washed four times with the provided wash buffer concentrate diluted in distilled water. The detection antibody, a biotinylated polyclonal antibody for porcine IFN-γ, diluted in the provided Dilution Buffer 1 (incubation period overnight at 2-8°C), and the Streptavidin-Alkaline Phosphatase, diluted in the provided Dilution Buffer 2 (incubation period 2 hours at room temperature), were added. Every incubation period was followed by four washing steps. The provided BCIP/NBT Chromogen was used as substrate solution (incubation period 1 hour at room temperature). Colour development was stopped by washing with distilled water. The plate was left aside to air dry. Spots were counted by an ELISpot reader (AID iSpot FluoroSpot Reader System, AID Autoimmun Diagnostika GmbH).

3.4. Statistical analysis

Statistical analysis was done with MedCalc® version 9.5.2.0. (MedCalc Software). Normal distribution of results was checked with the Kolmogorov-Smirnov test (p>0.05). According to the result, an ANOVA or the Kruskal-Wallis test were used for further analysis. Further tests were mentioned if used. Box and whisker plots were mainly used for presentation of results.

3.5. Animals and samples

Examinations were carried out with blood samples collected from non-vaccinated/non- infected, non-vaccinated/infected, US-vaccinated and EU-vaccinated sows. No animal experiment was done. Blood samples were taken in the course of routine diagnosis in stocks. Blood samples for the IFN-γ-RA using Li-Heparin as an anticoagulant and serum samples for SNT, PCR and antibody ELISA were collected. Blood samples were taken from V. jugularis. In total, 458 pigs of 49 stocks were tested. The sampling plan compromised ten blood samples per herd randomly collected from sows with different numbers of parity. Thirty-two blood samples were excluded from testing because the lack of or coagulated sample material.

4. Results

4.1. Analysis of IFN-γ-standards of two test kits

ODs for different concentrations of IFN-γ standards were examined. Kits from two manufacturers (A and B) were included (Fig. 2a-d). The minimum of detectable IFN-γ-standard as well as interplate and interday variation of standard curves were analysed. For the reason of comparability of kit A and B a maximum standard of 1000 pg/ml was used in the upcoming graphical representations.

According to the manufacturer's instructions, the standard of kit A was log2-diluted from 4000 to 62.5 pg/ml, whereas in kit B it was log10-diluted and ranged from 1000 to 10 pg/ml. A similar OD range at low IFN-γ-concentrations was detected in both kits. Regarding interplate and interday variation, kit B showed a greater interday variation than kit A (F-test for standard with IFN-γ-concentration of 1000 pg/ml, $P<0.05$).

Kit A IFN-γ-standard was tested at concentrations of 10, 100 and 1000 pg/ml in order to compare the detectability (Fig. 3). Kit A was unable to discriminate the lowest concentration (10 pg/ml) from the negative standard. Thus a higher detectability was observed for Kit B.
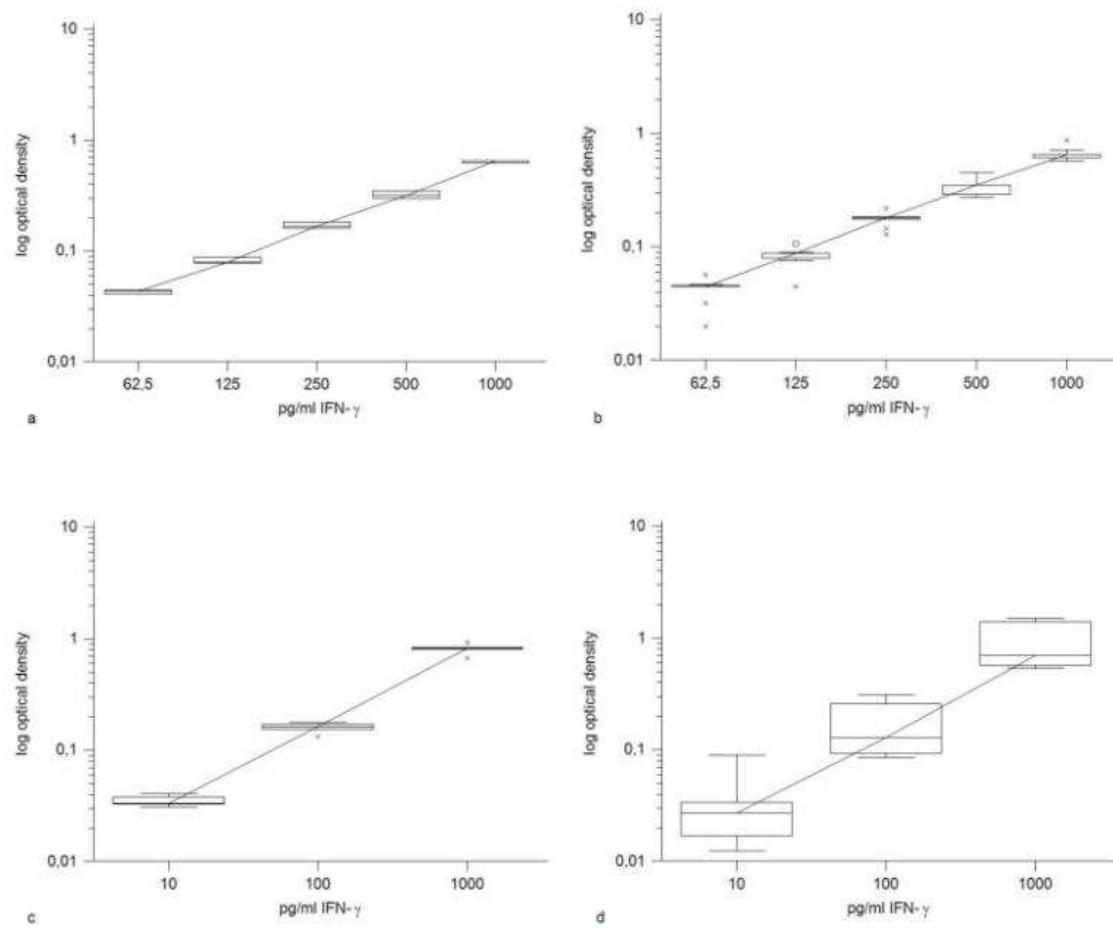
Figure 2: Optical densities of different IFN-γ-standard concentrations.

Source: Own presentation

Interplate (left) and interday (right) variation for test kit A (a,b) and B (c,d) are shown. Standards provided with the respective kit were used at prescribed concentrations. For interplate and interday variation, six and ten tests per concentration were used.
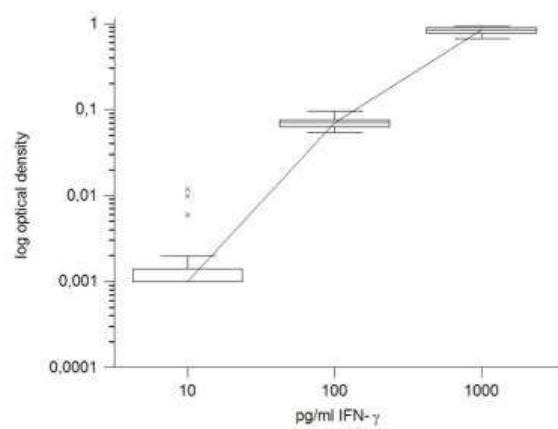

Figure 3: Optical densities of kit A IFN-γ-standard in kit B IFN-γ-standard concentrations.

Source: Own presentation

4.2. Titration of stimulation control

SC was implemented to confirm the viability and ability of PBMC to produce IFN-γ and to serve as a positive control to express the pathogen-specific IFN-γ-response as percent. SC-IFN-γ-reactivity should be as close as possible to that of positive blood samples to pathogen-specific antigens. If IFN-γ-reactivity to SC is too strong, the reaction has to be stopped before a sufficient reactivity of blood samples to pathogen-specific antigens developed. In order to assess the optimal concentration of SC, blood samples of 30 sows from three different stocks were stimulated with SC at 0.6, 0.3 and 0.17 µg/ml (Fig. 4). A concentration of 0.6 µg/ml resulted in IFN-γ-reactivity well distributed in the measuring range of the photometer. Twenty-three percent (kit A) and 10% (kit B) of the samples showed a reactivity below 0.4 (OD, validation criterion), whereas at a concentration of 0.3 µg/ml, 73% (kit A) and 60% (kit B) of the samples failed the criterion. Kit B showed a significantly stronger reactivity at 0.6 µg/ml (ANOVA, $P<0.05$), however, a significantly higher OD was observed for PBS, too. 37% of samples in kit B and only 7% in kit A failed the validation criterion $OD_{PBS}<0.2$.
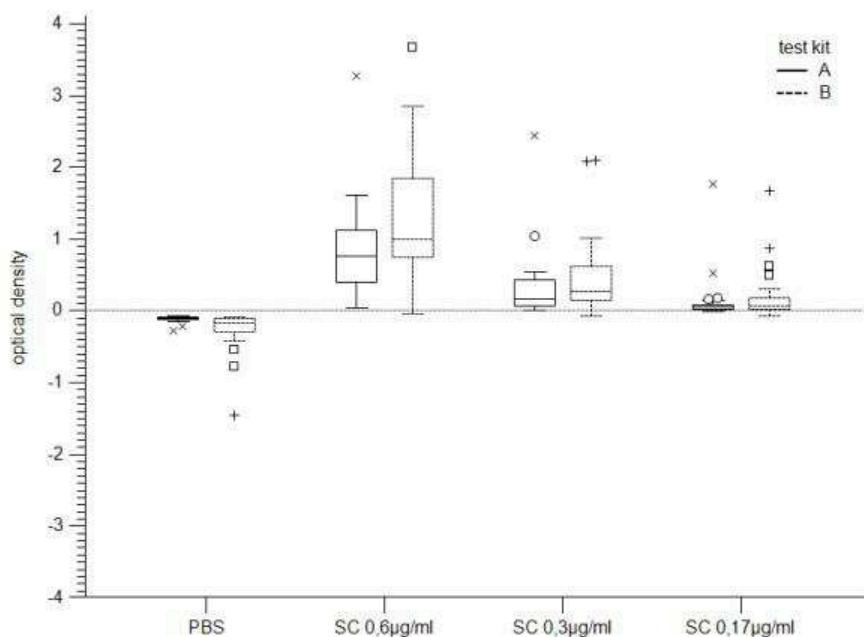

Figure 4: Quantification of IFN-γ after stimulation of Li-heparin-stabilized blood samples with different concentrations of pokeweed mitogen (SC).

Source: Own presentation

Kit A and B ($n_{stock}=3$, $n_{sow}=30$) were used. Stimulation with PBS served as a blank.

4.3. Reactivity against MARC145/FCS (control antigen)

Aside from an unspecific IFN-γ-reactivity in unstimulated blood samples (PBS) in kit B, an additional unspecific IFN-γ-reactivity against the control antigen (MARC145/FCS) was observed in both kits (Fig. 5). Blood samples of 20 sows from four different stocks were examined for reactivity against MARC145/FCS. The validation criterion OD_{PBS} <0.2 was not applied. Differences for MARC145/FCS between kits A and B were not significant (ANOVA, $P>0.05$).

To analyse the components of the control antigen for unspecific IFN-γ-reactivity, blood samples from 20 sows from two different stocks were examined for reactivity to MARC145/FCS, MARC145/PBS, PBS/FCS and EMEM w/o FCS (Fig. 6). FCS was responsible for the unspecific IFN-γ-response. This analysis was only performed in kit B.

In a second series of tests FCS was replaced by HS. Twenty blood samples of sows from two different stocks were examined (Fig. 7). Unspecificity was caused by FCS but was not detected for HS, however, differences were not significant (ANOVA, $P>0.05$)
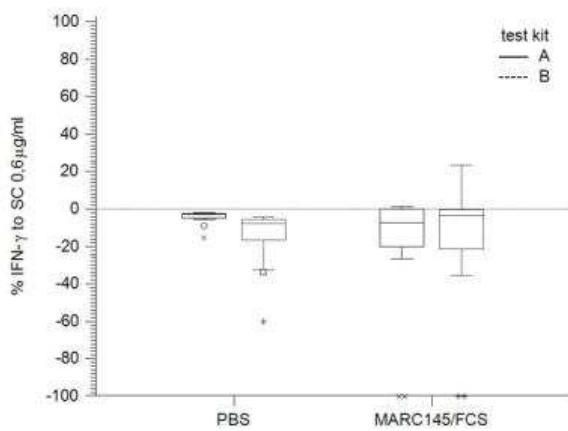
Figure 5: Unspecific reactivity in IFN-γ-RA (kit A and B).

Source: Own presentation

Stimulation of Li-heparin-stabilized blood samples with PBS and MARC145/FCS ($n_{stock}=4$, $n_{sow}=20$). The IFN-γ-reactivity was expressed as a percentage of SC and the maximum reactivity was restricted to 100%. $\%OD_{PBS}$ and $\%OD_{MARC145/FCS}$ were multiplied by -1. The validation criterion OD_{PBS} <0.2 was not applied.
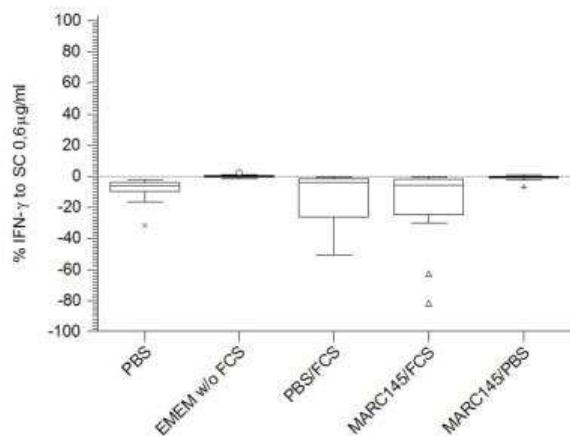
Figure 6: Analysis of MARC145/FCS components for unspecific IFN-γ-reactivity in test kit B (n_{stock}=2, n_{sow}=20).

Source: Own presentation

The IFN-γ-reactivity was expressed as a percentage of SC and the maximum reactivity was restricted to 100%. Values of negative controls were multiplied by -1. The validation criterion OD_{PBS} <0.2 was not applied. PBS/FCS = PBS supplemented with 10% FCS, MARC145/PBS = MARC145 prepared in PBS instead of EMEM/FCS10%.
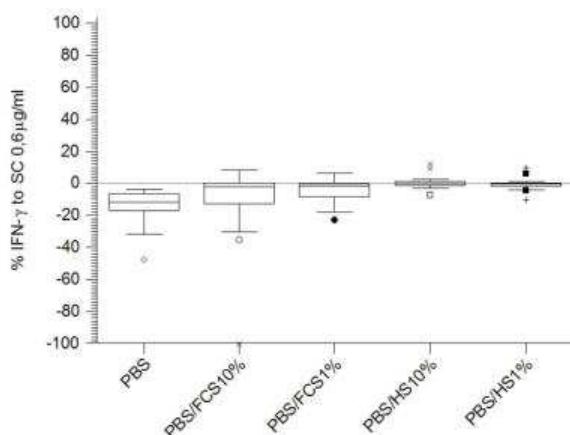
Figure 7: Analysis of HS as a substitute for FCS in test kit B (n_{stock}=2, n_{sow}=20).

Source: Own presentation

IFN-γ-reactivity was expressed as a percentage of SC and the maximum reactivity was restricted to 100%. Values of negative controls were multiplied by -1. The validation criterion OD_{PBS} <0.2 was not applied. PBS/FCS10% = PBS supplemented with 10%FCS; PBS/FCS1% = PBS supplemented with 1%FCS; PBS/HS10% = PBS supplemented with 10%HS; PBS/HS1% = PBS supplemented with 1%HS.

4.4. IFN-γ-reactivity against PRRSV-specific and control antigens

Subsequent experiments were performed with kit A because of a lower variation of interday variance and less frequently observed IFN-γ-responses in unstimulated blood samples (PBS). In order to circumvent the use of FCS, cell lysates of PRRSV-infected and non-infected MARC145-cells had been produced. The optimal dilution of these antigens for stimulation of blood samples was determined in PRRSV-US-vaccinated (Fig. 8a) and PRRSV-EU-vaccinated (Fig. 8b) stocks. Blood samples from nine US-vaccinated and eight EU-vaccinated sows were stimulated with final antigen dilutions of 1:150, 1:750 and 1:1500 (pre-dilutions 1:10, 1:50 and 1:100). In particular, the dilution of US-antigen was investigated with blood samples of US-vaccinated animals and the dilution of EU-antigen with blood samples of EU-vaccinated animals. A remarkably specific IFN-γ-response was observed for EU- and US-antigens in EU- and US-vaccinated herds, respectively. However, no significant differences were observed between the dilutions (ANOVA, $P>0.05$), although a tendency of a reduced reactivity upon dilution was evident at least for the US-antigen. Therefore in the following experiments two dilutions of the antigens (1/150 and 1/750) were applied.
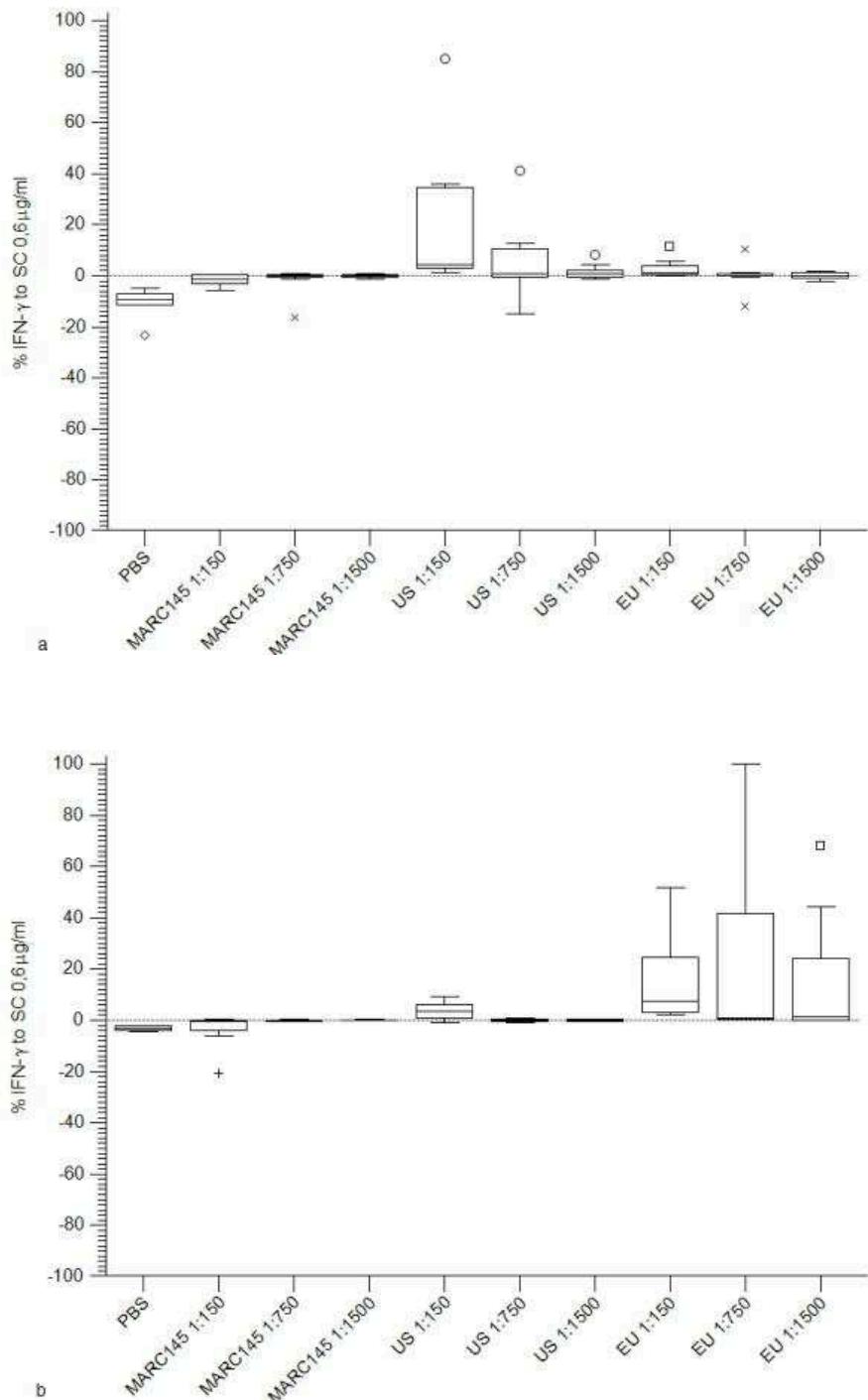
Figure 8: Effect of PRRSV-EU- and -US-antigen dilution on IFN-γ-reactivity.

Source: Own presentation

Li-heparin-stabilized blood samples from sows originating from US- (a, $n_{stock}=2$, $n_{sow}=9$) and EU-vaccinated (b, $n_{stock}=1$, $n_{sow}=8$) herds were stimulated with dilutions of antigens. IFN-γ-reactivity was expressed as a percentage of SC and the maximum reactivity was restricted to 100%. %OD_{PBS} and %$OD_{MARC145}$ were multiplied by -1.

4.5. IFN-γ-profiles of sows

In order to determine the feasibilty of the PRRSV-IFN-γ-RA sows from herds with a different history of PRRSV-infection and vaccination were tested. Therefore, blood samples from non-vaccinated/non-infected, non-vaccinated/infected, US-vaccinated and EU-vaccinated sows were tested. As indirectly indicated by the litter number different age groups of sows were considered.

A total of 261 sows from 27 stocks were examined. Validation criteria led to the exclusion of 13% of samples (17 samples with OD_{SC} <0.4 and 17 samples with OD_{PBS} ≥0.2). Annex 4 gives an overview of the analysed and invalid samples.

An estimate on specificity of the IFN-γ-test was assessed by testing non-vaccinated/non-infected stocks (Fig. 9a). Blood samples from 28 sows from three stocks were investigated and 7% (two samples from one stock) of the samples were invalid because OD_{SC} <0.4. A slight reactivity was observed against MARC145 at a dilution of 1:150. The maximum reactivity for US- (1:150) and EU-antigen (1:150) was 12.6% and 8%, respectively.

Thirty sows from three non-vaccinated/infected stocks were studied for their IFN-γ-reactivity (Fig. 9b). 20% of the samples failed the validation criteria. Reactivity against MARC145 was comparable to those in Fig. 9a. A stronger reactivity was observed against the EU-antigen compared to the US-antigen. The EU-antigen diluted 1:150 resulted in a significantly stronger IFN-γ-reactivity compared to a 1:750 dilution (ANOVA, P<0.05).

Eighty-one sows from eight US-vaccinated stocks were examined in IFN-γ-RA (Fig. 9c). The validation criteria led to the exclusion of 19.8% of samples from evaluation because of ten samples with OD_{SC} <0.4 and six samples with OD_{PBS} ≥0.2. The US-antigen induced IFN-γ-reactivity and differences between US 1:150 and 1:750 were significant (Kruskal-Wallis test, P<0.05). EU-antigens remained at a low level except for single reactors.

The highest level of IFN-γ-reactivity was observed for EU-antigen in 122 sows from 13 EU-vaccinated stocks (Fig. 9d). Eight percent didn't fulfil the validation criteria (three samples with OD_{SC} <0.4 and seven samples with OD_{PBS} ≥0.2). Differences between EU 1:150 and EU 1:750 were significant (Kruskal-Wallis test, P<0.05). US-antigens remained almost negative with single samples showing low IFN-γ.
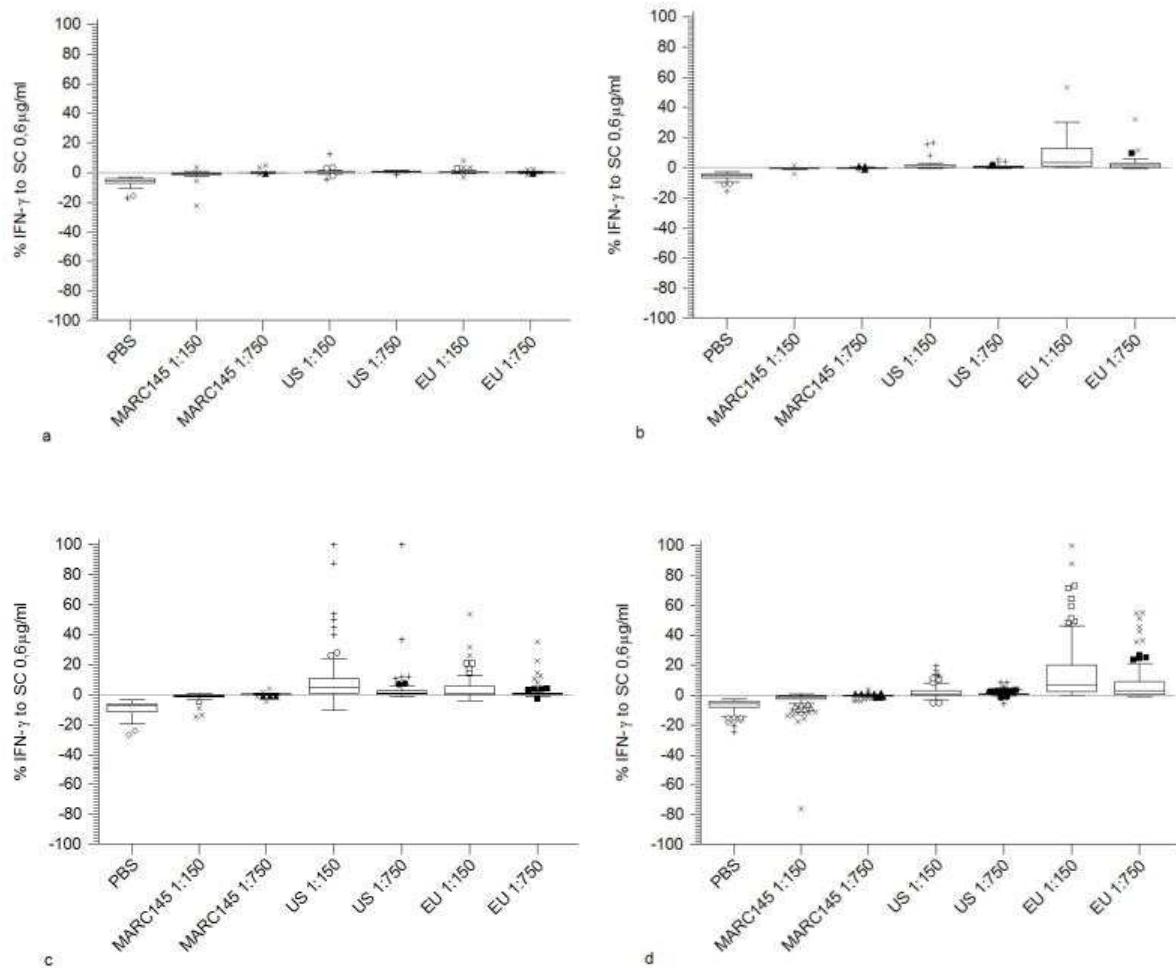
Figure 9: IFN-γ-reactivity of sows against vaccine derived antigens.

Source: Own presentation

Sows from non-vaccinated/non-infected (a, $n_{stock}=3$, $n_{sow}=26$), non-vaccinated/infected (b, $n_{stock}=3$, $n_{sow}=24$), US-vaccinated (c, $n_{stock}=8$, $n_{sow}=65$) and EU-vaccinated (d, $n_{stock}=13$, $n_{sow}=112$) herds. IFN-γ-reactivity was expressed as a percentage of SC and the maximum reactivity was restricted to 100%. %OD_{PBS} and %$OD_{MARC145}$ were multiplied by -1.

4.6. Determination of appropriate cut-offs

In order to define positive and negative results, non-vaccinated/non-infected stocks were used to set a cut-off value for the evaluation of infected and/or vaccinated stocks. Arithmetic means and standard deviations (s) of US and EU 1:150 were consulted for calculation of possible cut-off values. Table 4 gives an overview of calculations for single, two- and three-fold standard deviations. Considering the non-vaccinated/non-infected animals to be negative, a cut-off value of 10% of SC seems to be an appropriate criterion for the differentiation of positive and negative samples. Table 5 (see page 35) gives an overview of the IFN-γ-positivity in the tested stocks on the basis of the cut-off value.

Table 4: Determination of cut-off values with single, two- and three-fold standard deviations (% to SC).

	arithmetic mean+1s	arithmetic mean+2s	arithmetic mean+3s
US1:150	3.5342	6.3492	9.1642
EU1:150	2.6501	4.5848	6.5195

Source: Own presentation

4.7. IFN-γ-reactivity to field isolates

So far, reactivity against EU- and US-antigens from vaccine virus was tested. In this study four antigens from wild-type isolates were included. Nineteen non-vaccinated/non-infected sows from two stocks, 29 sows from three US-vaccinated stocks and 47 sows from five EU-vaccinated stocks were examined in IFN-γ-RA (Fig. 10). Three US-vaccinated sows from one stock and four EU-vaccinated sows failed OD_{SC} <0.4 and one EU-vaccinated sow failed OD_{PBS} ≥0.2 so that 8.4% of samples were excluded from evaluation. An increased unspecific reactivity against non-infected PAM was noticed. PAM reactivity was subtracted from reactivity to antigens from wild-type isolates. These antigens induced an IFN-γ-response in vaccinated sows comparable to that of vaccine-derived antigens although virus titers of wild-type isolates were about a thousand times lower. IFN-γ-reactivity to field isolates remained weak in non-vaccinated/non-infected herds. No significant differences existed between the four field isolates except of FI I being different from FI II and FI III (ANOVA, P<0.05) in EU-vaccinated stocks. Differences between EU-antigen and field isolates were not significant in US- and EU-vaccinated stocks as well.
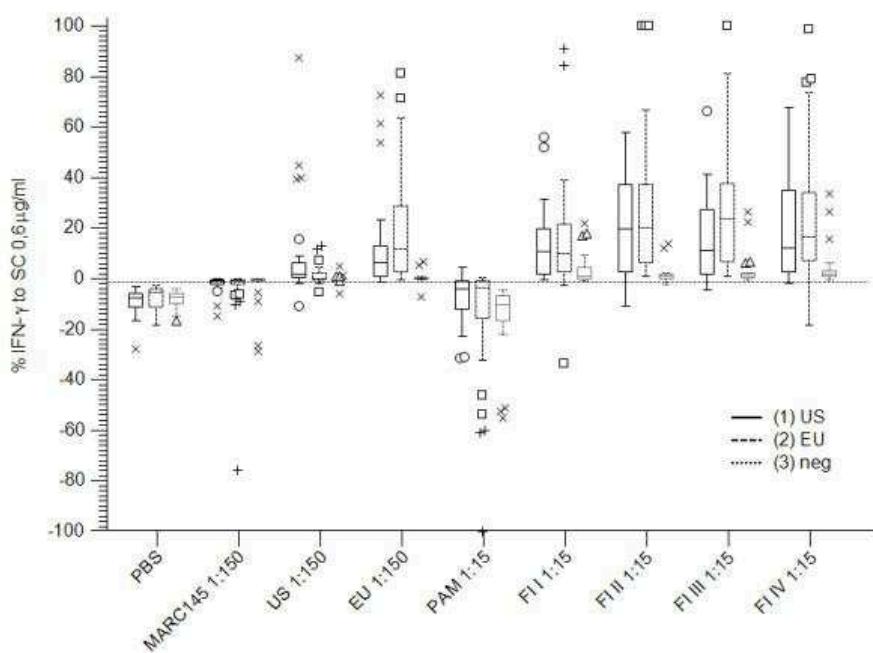
Figure 10: IFN-γ-reactivity of sows against field virus derived antigens.

Source: Own presentation

This Figure shows results of sows from non-vaccinated/non-infected (n_{stock}=2, n_{sow}=19), US-vaccinated (n_{stock}=3, n_{sow}=26) and EU-vaccinated (n_{stock}=5, n_{sow}=42) herds. The IFN-γ reactivity was expressed as a percentage of SC and the maximum reactivity was restricted to 100%. $\%OD_{PBS}$, $\%OD_{MARC145}$ and $\%OD_{PAM}$ were multiplied by -1.

4.8. Comparison of IFN-γ-test with other diagnostic tests

Antibody ELISA and SNT were performed in parallel to IFN-γ-RA. A total of 244 sows from nine US- and 14 EU-vaccinated stocks, three non-vaccinated/infected stocks and three non-vaccinated/non-infected stocks were included. Annex 5 gives an overview of the comparison of ELISA, SNT and IFN-γ-RA results.

4.8.1. Comparison with antibody ELISA

No correlation was found between ELISA antibodies and IFN-γ-reactivity to US 1:150 (Spearman correlation coefficient rho -0.0237; Fig. 11a). A slight positive correlation between ELISA antibodies and IFN-γ-reactivity to EU 1:150 was observed (Spearman correlation coefficient rho 0.537; Fig. 11b).

4.8.2. Comparison with serum neutralisation test

Animals were classified as SNT-positive or -negative for each viral genotype (PRRSV-US and -EU, respectively). Subsequently, animals were allocated to four groups SNT(US-/EU-), SNT(US+/EU+), SNT(US+/EU-) and SNT(US-/EU+) (Fig. 12). In group SNT(US-/EU-) only single samples developed an IFN-γ-response, whereas a striking reactivity to both US- and EU-antigens was observed in group SNT(US+/EU+). IFN-γ-reactivity to US-antigen in group SNT(US+/EU-) and to EU-antigen in group SNT(US-/EU+) indicated a relationship between IFN-γ-response and SNT. IFN-γ-response to US-and EU-antigen was significantly different in groups SNT(US+/EU-), SNT(US-/EU+) and SNT(US+/EU+) (Kruskal-Wallis test, $P<0.05$). Irrespective of EU-specific neutralising antibodies, SNT-US-negative and -positive groups were different to each other regarding IFN-γ-reactivity to US-antigen. Vice versa, SNT-EU-negative and -positive groups were different in the IFN-γ-response to the EU-antigen.

According to the IFN-γ-positivity of sows tested in IFN-γ-profiles (table 5), table 6 gives an overview of the rate of SNT >2 of sows tested in the present examination.
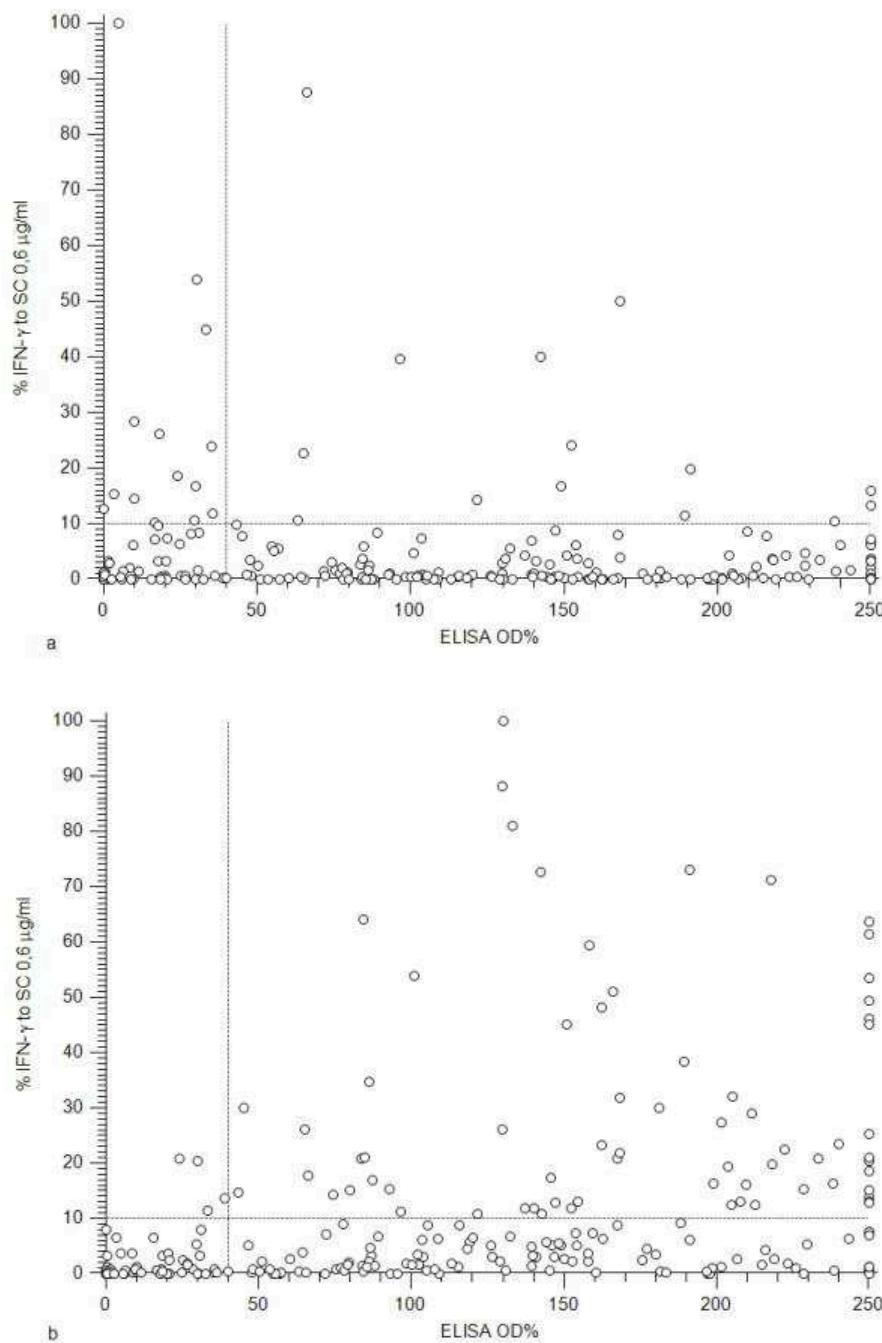
Figure 11: Comparison of antibody ELISA and IFN-γ-response.

Source: Own presentation

IFN-γ-response towards US 1:150 (a) and EU 1:150 (b) in $n_{sow}=248$ ($n_{stock\ (non\text{-}vaccinated/non\text{-}infected)}=3$, $n_{stock\ (non\text{-}vaccinated/infected)}=3$, $n_{stock\ (US\text{-}vaccinated)}=9$, $n_{stock\ (EU\text{-}vaccinated)}=14$). IFN-γ-reactivity was expressed as a percentage of SC and the minimum and maximum reactivity was restricted to 0%, 100% (IFN-γ-RA) and 250% (antibody ELISA). Cut-off values of 10% (IFN-γ-RA) and 40% (antibody ELISA) are indicated by horizontal and vertical lines, respectively.
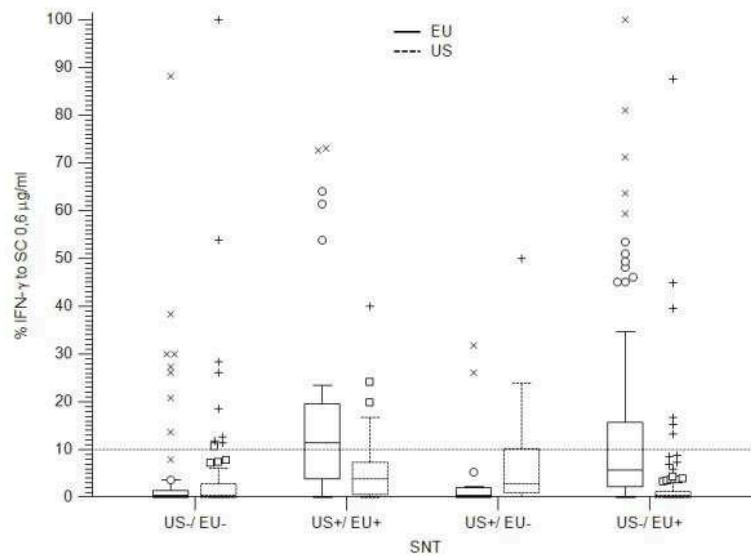
Figure 12: Comparison of SNT and IFN-γ-response.

Source: Own presentation

IFN-γ-response towards US 1:150 and EU 1:150 in n_{sow}=248 ($n_{stock\ (non-vaccinated/non-infected)}$=3, $n_{stock\ (non-vaccinated/infected)}$=3, $n_{stock\ (US-vaccinated)}$=9, $n_{stock\ (EU-vaccinated)}$=14). Animals were classified as SNT-positive or -negative for each PRRSV-EU and -US ($n_{sow(SNT(US-/EU-))}$=71, $n_{sow(SNT(US+/EU+))}$=50, $n_{sow(SNT(US+/EU-))}$=23, $n_{sow(SNT(US-/EU+))}$=104). Subsequently animals were allocated to four groups. IFN-γ-reactivity for each group was determined. IFN-γ-reactivity was expressed as a percentage of SC and minimum and maximum reactivity in IFN-γ-RA was restricted to 0% and 100%. The cut-off value of 10% in IFN-γ-RA is indicated with a horizontal line.

Table 5: IFN-γ-positivity in the different herds on the basis of a 10% cut-off value

	IFN-γ-reactivity to US 1:150	IFN-γ-reactivity to EU 1:150
US-vaccinated	28%	17%
EU-vaccinated	5%	43%
Non-vaccinated/infected	8%	29%

Source: Own presentation

Table 6: Rate of positive SNT (>2) in the different herds

	US-SNT	EU-SNT
US-vaccinated	60%	43%
EU-vaccinated	19%	90%
Non-vaccinated/infected	8%	50%

Source: Own presentation

4.9. Comparison of IFN-γ-reactivity considering different age groups

The litter number per sow was used as an indirect correlate of age. IFN-γ-reactivity to US 1:150 and EU 1:150 was examined (Fig. 13a-d). Seventy-nine US-vaccinated (Fig. 13a), 120 EU-vaccinated (Fig. 13b), 24 non-vaccinated/infected (Fig. 13c) and 26 non-vaccinated/non-infected sows (Fig. 13d) were assigned to three groups of litter no. "1./2." including gilts (litter no. "0."), "3./4." and "5./6.". The last group also included sows with litter no. ">6.".

In US-vaccinated stocks 14, 27 and 34 sows were assigned to the three groups. IFN-γ-reactivity against both antigens (US + EU) increased with the litter number, but differences against EU-antigen were significant between groups "1./2." and "5./6." (ANOVA, $P<0.05$).

Forty-five, 23 and 51 sows were included in groups in EU-vaccinated stocks. IFN-γ-reactivity didn't vary with the litter number.

Non-vaccinated/infected herds with 6, 12 and 6 sows per group showed the highest IFN-γ-response to EU-antigen in the age group with one or two litters. Differences were again not significant (ANOVA, $P>0.05$).

Non-vaccinated/non-infected herds remained unobtrusive.
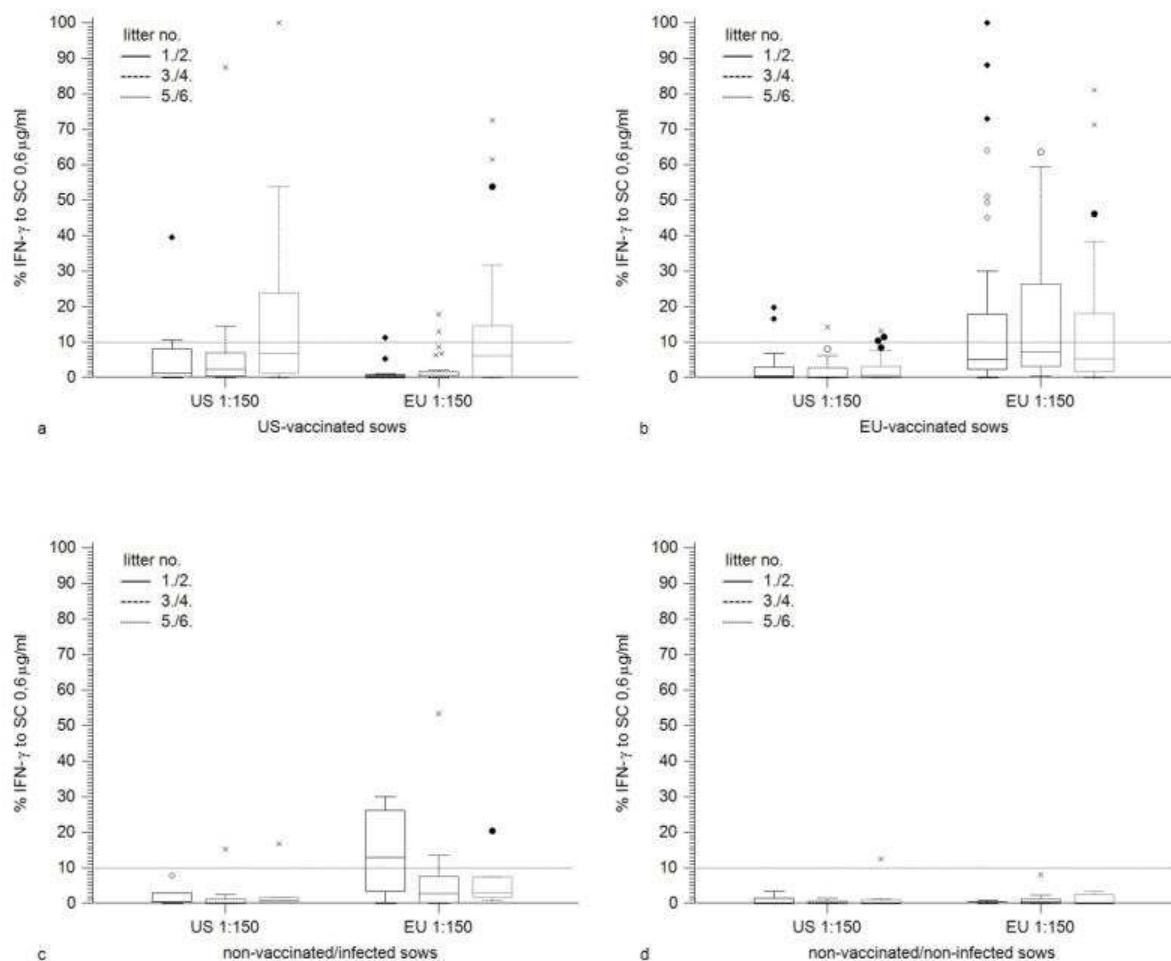
Figure 13: Effect of litter number per sow on IFN-γ-reactivity.

Source: Own presentation

IFN-γ-reactivity to US and EU 1:150 in US-vaccinated (a, n_{stock}=10, n_{sow}=79), EU-vaccinated (b, n_{stock}=15, n_{sow}=120), non-vaccinated/infected (c, n_{stock}=3, n_{sow}=24) and non-vaccinated/non-infected sows (d, n_{stock}=3, n_{sow}=26) is shown. IFN-γ-reactivity was expressed as a percentage of SC. Minimum and maximum reactivity in IFN-γ-RA was restricted to 0% and 100%. The cut-off value of 10% in IFN-γ-RA is indicated with a horizontal line.

4.10. Comparison of IFN-γ-reactivity with PBMC-counts in whole blood samples

For comparison of IFN-γ-reactivity with PBMC-counts in whole blood samples, the amount of IFN-γ produced in stimulation control was used. PBMC in Li-Heparin stabilized whole blood samples of 257 sows were counted. No correlation was found between IFN-γ-reactivity and the number of PBMC (Spearman correlation coefficient rho 0.0134; Fig. 14). It had to be noticed that in many samples the PBMC numbers lay below the physiological limits.
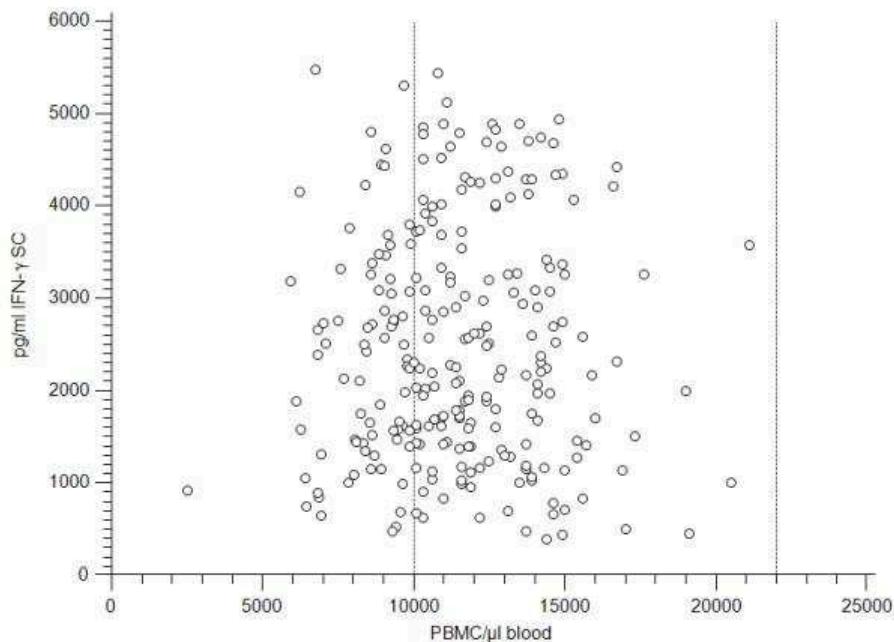
Figure 14: Effect of PBMC numbers on IFN-γ-reactivity of Li-Heparin stabilized blood samples to stimulation control (n_{stock}=31, n_{sow}=257).

Source: Own presentation

Reactivity towards SC is presented in pg/ml IFN-γ compared to the number of PBMC/µl whole blood. Physiological limits of number of PBMC (10.000-22.000/µlblood) are indicated with vertical lines.

4.11. Stability studies of antigens

For evaluation of stability, MARC145, US- and EU-antigen were stored at 2 - 8°C and -20°C for 2, 4 and 8 weeks and tested in parallel. Storage at -80°C served as reference. Seventeen EU-vaccinated sows from two stocks were included in this experiment. Stability of antigens was examined in general (Fig. 15a) and for EU-antigen separately per animal (Fig. 15b). Storage conditions had no significant effect on IFN-γ-reactivity regarding the EU-antigen (Kruskal-Wallis, P>0.05). Figure 15b shows that EU-antigens induced comparable IFN-γ-reactivity in the animals independent from storage conditions.

4.12. IFN-γ-ELISpot

IFN-γ-RA was compared with IFN-γ-ELISpot. The number of PBMC detected in the ELISpot is presented in Figure 18. Positive reacting cells could not be quantified until SC 0.1 μg/ml. PBMC from 24 sows originating from three EU-vaccinated stocks reacted positive to stimulation with EU-antigens. Stimulation with US-antigens led to IFN-γ-production in some cells as well. Reactivity to EU 1:150 is significantly different to EU 1:750 and to the US-antigens (ANOVA, P<0.05). Figure 19 compares data of IFN-γ-RA and IFN-γ-ELISpot by correlation. A slight positive correlation between both tests was observed (Spearman correlation coefficient rho 0.59).
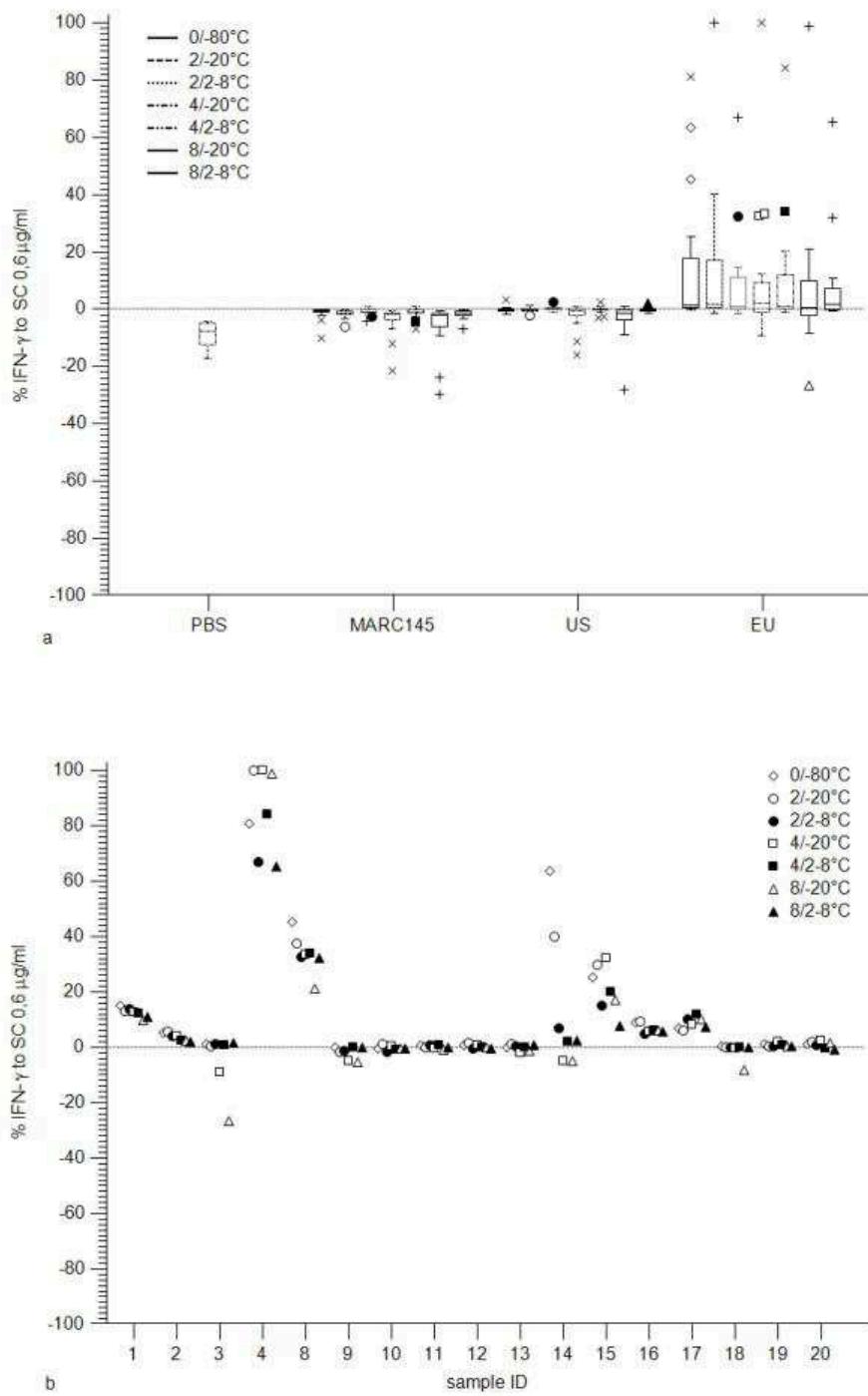
Figure 15: Effect of storage conditions for antigens on IFN-γ-reactivity.

Source: Own presentation

Reactions in EU-vaccinated stocks (n_{stock}=2, n_{sow}=17) to antigens 1:150 in general (a) and per animal to EU-antigen (EU 1:150) (b) are shown. The validation criteria SC≥0.4 and OD_{PBS}<0.2 were not applied. IFN-γ-reactivity was expressed as a a percentage of SC and maximum reactivity was restricted to 100%. $\%OD_{PBS}$ and $\%OD_{MARC145}$ were multiplied by -1.
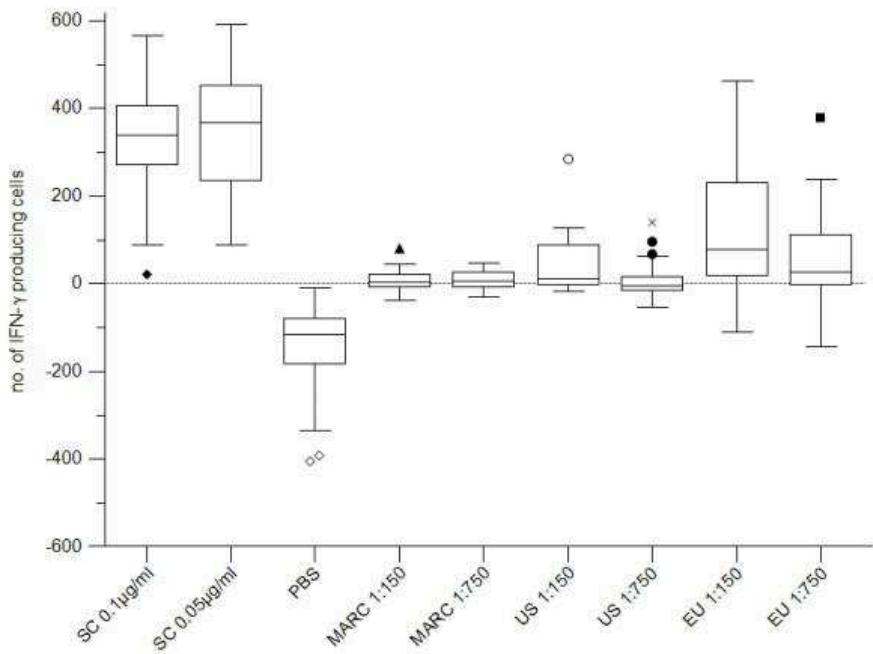
Figure 16: Quantification of IFN-γ-producing cells in an ELISpot (n_{stock}=3, n_{sow}=24). Source: Own presentation

$PBMC_{PBS}$ was subtracted from $PBMC_{MARC145}$. $PBMC_{MARC145}$ was subtracted from $PBMC_{US}$ and $PBMC_{EU}$. $PBMC_{PBS}$ and $PBMC_{MARC145}$ were multiplied by -1.
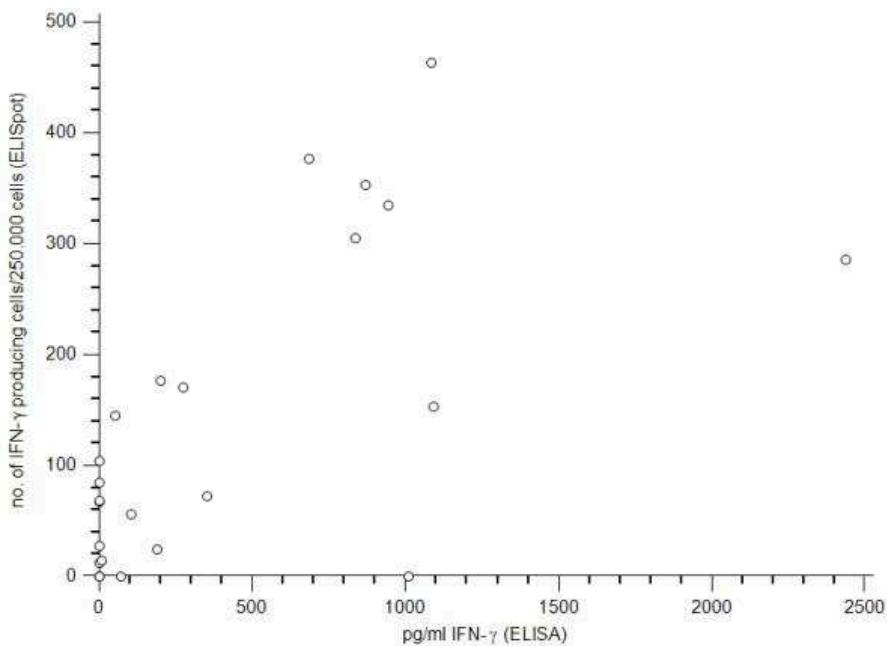
Figure 17: Comparison of IFN-γ-RA and IFN-γ-ELISpot (n_{stock}=3, n_{sow}=24).
Source: Own presentation

5.   Discussion

5.1.   Preliminary considerations

PRRS is a major problem in swine industry. Reproductive failure in sows and respiratory tract disease in growing pigs results in tremendous economic losses (Holtkamp et al., 2013; Neumann et al., 2005). It is further complicated by secondary bacterial infection in growing and fattening pigs (Drew, 2000; Zimmerman et al., 2012) that require antibiotic treatment. Consequently, concerns about the development of antibiotic resistance of bacteria rose. Efforts to reduce the amount of antibiotics in veterinary medicine are confirmed by the 16th law amending the German medicine act (AMG) relating to the manufacture of medicines in Germany that will come into force on 1th April 2014 (Anonymous, 2013a). Vaccination against PRRSV might contribute to the reduction of antibiotic treatments. Several commercial attenuated and inactivated vaccines are currently in use. However, PRRSV-related problems are limiting the efficacy of vaccination:

(1) PRRS-infection results in immunity to the homologous challenge that lasts for at least 600 days (Lager et al., 1997a). Ideally a vaccine should achieve similar protection. This kind of protection might cover antigenically related virus, but it decreases against more distantly related (heterologous) challenge virus (Labarque et al., 2004). Field virus strains are an unknown variable and new virus variants are continuously emerging (Labarque et al., 2004). This situation explains why vaccination with attenuated virus only reduces the level of viremia and severity of clinical disease.

(2) Vaccination of piglets in the second or third week of life is recommended. The same vaccine for both sows and piglets is used. But if vaccination of sows induces sufficient neutralisation titers, these antibodies are transferred to piglets and neutralise the vaccine virus. Indeed passively transferred neutralising antibodies blocked infectivity of the homologous virus (Osorio et al., 2002). Assessment of vaccine-virus-specific neutralising antibody titers in piglets before vaccination might be helpful in order to pre-estimate the time of vaccination.

(3) If vaccination of sows fails to induce homologous neutralising antibodies, newborn piglets are not protected by maternally derived antibodies. In EU-

vaccinated herds with ongoing PRRSV-EU-circulation in weaned piglets a significant lower level of EU-neutralising antibodies are detected than in EU-vaccinated herds without detection of PRRSV-EU in weaned piglets (Böttcher et al., accepted). Additionally, in those EU-vaccinated herds with ongoing virus circulation, sows of first and second parity showed a significantly lower level of neutralising antibodies as compared to older sows. This finding indicates a possible gap in herd immunity (Böttcher et al., accepted).

Insufficient immune responses and gaps in immunity are not yet encountered systematically as a possible cause of vaccine failure.

Efficacy of vaccination should be controlled by measuring the immune response after vaccination. Routine diagnosis of PRRS is based on PCR and antibody-ELISA. Both tests are of limited value in assessing the immune response after vaccination. Specifically, the ELISA detects non-neutralising antibodies that are directed against the nucleoprotein. Detection of wild-type PRRSV by PCR in vaccinated animals indicates an insufficient immune response, but the damaging event already occurred.

In contrast to that, neutralising antibodies and pathogen-specific IFN-γ-responses would be appropriate correlates of protective immunity after vaccination against PRRSV. As SNT-titers are only reflecting the humoral immune response, vaccine-virus-specific IFN-γ-reactivity mirrors cellular immunity. Analysis of both allows a more meaningful diagnosis of PRRSV-immunity.

5.2.   Complexity of IFN-γ-recall-assay (RA)-validation

The OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2013 (Anonymous, 2013b; online English version) provides guidelines for development and validation of diagnostic tests. These guidelines are helpful for the validation of serological tests, but some problems rose regarding the validation of IFN-γ-RA:

(1) In case of serological tests a panel of sera with known status, probably stored at -80°C, might be used. However, due to the reliance of IFN-γ-RA on stimulation of viable PBMC, blood samples have to be collected freshly for each testing. As a routine diagnostic laboratory without an experimental animal facility, animals with a defined status were not available. Only samples from the field were accessible. Validation relied on blood samples from routine

diagnostic submissions. Animals were defined by the infection status of herds regarding vaccination, presence or absence of antibodies in ELISA and SNT as well as detection of PRRSV by PCR.

(2) Common diagnostic tests only define results as positive or negative. However, tests for immunity should provide quantitative outputs. The amount of IFN-γ is frequently expressed e.g. as pg/ml or in case of ELISpot as spots per tested cells. This kind of quantification does not reflect the animal's general ability to produce IFN-γ and the quality of the PBMC in blood samples to produce IFN-γ. A negative value after stimulation with PRRSV-antigen might reflect a general inability of PBMC to produce IFN-γ. A SC had to be included as a reference and an appropriate concentration had to be determined in order to express the pathogen-specific IFN-γ-reactivity as a percentage of the SC.

(3) Cut-off values are imperative for each diagnostic tool. At this early stage of validation no gold-standard is available for analysis strategies. Additionally, a cut-off for IFN-γ-positivity that differentiates infected from negative animals does not necessarily correspond to the level of protection. Therefore only a scale of IFN-γ-reactivity was provided on which the latter might be selected.

(4) Aside from SC additional appropriate controls had to be chosen. It has to be kept in mind that animals are repeatedly vaccinated with vaccines produced in cell-culture. Contaminants in vaccines might induce unspecific IFN-γ-reactivity and has to be ruled out with a cell control.

(5) As mentioned previously, IFN-γ-testing requires viable cells. The time span between sampling and testing is critical. The stimulation of cells was performed within eight hours after blood collection. Blood samples were transported directly from the farm to the lab. Testing of blood samples not later than 24 hours after collection is crucial for the implementation of an IFN-γ-RA in a routine diagnostic laboratory.

(6) The immune status of an animal changes in the course of infection or vaccination. Different levels of IFN-γ-producing cells are expected e.g. after primary infection/vaccination, after secondary (booster) infection/vaccination or after clearance of the virus as it is the case late after infection/vaccination, when only few memory cells are present in the circulation. This issue needs to be addressed in defined animal trials which are not addressed in the current study.

Despite this extensive list of problems a promising prototype of a PRRSV-IFN-γ-RA was provided and its general feasibility was demonstrated. A comprehensive validation considering the guidelines of the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2013 (Anonymous, 2013b; online English version) was not possible due to limitations of time, material and costs. Further examinations are necessary for improvement of the provided test system.

5.3.   Comparison of IFN-γ-RA and ELISpot

The IFN-γ-RA and the IFN-γ-ELISpot were compared. The following aspects were considered:

(1) The IFN-γ-ELISpot is five to ten-fold more expensive than the IFN-γ-RA.

(2) The IFN-γ-RA detects IFN-γ in plasma. Therefore it is sufficient to stimulated whole blood samples. In contrast to that, PBMC need to be purified, counted and set to a defined number of PBMC in case of ELISpot. In IFN-γ-RA the number of antigens (or e.g. the dilutions of antigens) might be easily extended without a dramatic increase of laboratory work. An increase of the number of antigens in ELISpot might require an additional PBMC-purification.

(3) The IFN-γ-ELISpot requires a longer incubation period during stimulation (20-24 hours) compared to IFN-γ-RA (16 hours).

(4) Photometers to read ODs of IFN-γ-RA are available in every routine diagnostic laboratory. An ELISpot-reader is required to count stained spots. Additionally, each plate needs to be adjusted and assessment of data takes more time.

(5) The detectability of the ELISpot is higher since single IFN-γ-producing cells are detected. This is exemplified by the SC: A concentration of 0.6 µg/ml was required for IFN-γ-RA whereas a lower concentration of 0.1 µg/ml allowed counting of single spots.

In summary, several advantages are in favour of IFN-γ-RA for routine use compared to IFN-γ-ELISpot. Nevertheless, the IFN-γ-ELISpot as a second test was established. The same blood samples were tested with IFN-γ-RA and ELISpot. Blood samples were prepared for IFN-γ-ELISpot as published (Dotti et al., 2013; Molina et al., 2008; Piras et al., 2005; Zuckermann et al., 2007). Similar to the results of Dotti et al. (2013) a good agreement between the IFN-γ-reactivity both in IFN-γ-RA and ELISpot was found. Results of Dotti et al. (2013) indicated a

lack of PRRSV-specific IFN-γ-response in infected pigs, whereas in the present examinations a clear IFN-γ-response to EU-antigen in EU-vaccinated herds was observed for several animals in IFN-γ-RA and ELISpot as well. Contrary, Díaz & Mateu (2005) rose concerns about the interpretation of IFN-γ-ELISpot in comparison to the IFN-γ-RA. They considered that no correlation exists between the number of IFN-γ-secreting cells in the ELISpot and IFN-γ-intensity in the recall assay. Furthermore, densities of seeded cells in the ELISpot need to be adapted with the age of tested animals because IFN-γ-frequencies of cytokine secreting cells may vary with the age (Díaz & Mateu, 2005).

The IFN-γ-RA was selected because of several advantages towards the ELISpot, which are less expenditure of time in sample preparation, inclusion of several antigens due to more sample material, better evaluability of raw data and lower costs of test material. Nevertheless, the IFN-γ-ELISpot probably should be taken into consideration as a further test for definition of cut-offs for protective IFN-γ-reactivity.

5.4. Selection of the test kit for the detection of IFN-γ

Two commercial test kits for the detection of IFN-γ in plasma of stimulated blood samples were included in this study. Both test kits provided standards. The standard of test kit A had to be diluted in 1/2-steps and covered the range from 4000 down to 62.5 pg/ml, whereas in kit B three concentrations 1000, 100 and 10 pg/ml had to be included. Kit A was unable to discriminate an IFN-γ-concentration of 10 pg/ml from the negative standard. Two disadvantages were observed for test kit B. Firstly, it showed a significantly higher interday variation compared to the interplate variation (Fig. 2, Chapter 4.1.). Secondly, unstimulated plasma samples (PBS) showed an increased reactivity than in kit A (Fig. 4, Chapter 4.2.). With regard to Figure 4 and the following figures, it should be kept in mind that undesirable reactivity as observed in PBS-control or against MARC145 are expressed as negative values (multiplied by -1), indicating that PBS- and MARC145-reactivity was subtracted from SC and specific antigens, respectively. An unspecific IFN-γ-reactivity in PBS-control was also noticed by Dotti et al. (2013). They explained this by a concurrent infection of animals with other pathogens. However, as IFN-γ-productivity in the PBS control in present examinations only was noticed in one of the two test kits it was assumed to be a test

specific problem. Indeed, kit B relies on two mouse MAb, one as capture- and the second as detection antibody. Consequently, anti-mouse IgG-antibodies in pig sera might bridge unspecifically between both MAbs, which results in a false positive signal. In contrast to that, kit A uses a mouse MAb as capture and polyclonal anti-IFN-γ-goat-IgG as detection antibody. Additionally, goat serum is added to the detection antibody. An important further question concerns the detectability of IFN-γ. A concentration of 62.5 pg/ml IFN-γ resulted in a net-reactivity of 40-50 mOD in test kit A (Fig. 2a and b). Dotti et al. (2013) presented their results of a PRRSV-IFN-γ-ELISA in mOD. They determined cut-offs by testing whole blood samples of SPF pigs. They scored samples positive if the OD in PRRSV-stimulated whole blood cultures was at least 50 mOD higher than in the corresponding mock-stimulated and unstimulated cultures. Unfortunately detectability was not assessed in that study. Mikkelsen et al. (2012) analysed the IFN-γ-reactivity against *Mycobacterium avium ssp. paratuberculosis* (MAP) in cattle. They expressed data as pg/ml. MAP non-infected herds were used to define cut-off values of ≥1000 pg/ml and <150 pg/ml for positive and negative control, respectively. IFN-γ-reactivity to specific antigens was corrected by subtraction of IFN-γ-response of PBS-control. Their results ranged from 1 to 10.000 pg/ml.

With detection of higher amounts of IFN-γ, Subharat et al. (2012) chose ng/ml for presentation of IFN-γ-quantity. They calculated IFN-γ-reactivity to MAP against a standard curve prepared with recombinant IFN-γ. The cut-off value for positive IFN-γ-reactivity was calculated from the mean and two standard deviations of the value for control animals and was set to 3.80 ng/ml IFN-γ. They gained maximum IFN-γ-reactivity of 40 ng/ml.

In their study of IFN-γ-reactivity against *Coxiella burnetii*, Roest et al. (2013) used a positive control (stimulation control) in the ELISpot assay as a reference for IFN-γ-responses in goats. IFN-γ-reactivity previously was corrected by subtraction of IFN-γ-response to the medium control.

According to Roest et al. (2013) SC was chosen as a reference for IFN-γ-reactivity to specific antigens in the present experiments. IFN-γ-standard in pg/ml was used for the calibration of the SC.

Kit A was selected for further IFN-γ-examinations. Standard series was well distributed within the measuring range of the photometer. Kit A further showed lower interday variation in standards and lower unspecific reactivity to PBS.

5.5.   Stimulation and PBS control

The SC was included for two reasons:

(1) confirmation of viability and ability of PBMC to produce IFN-γ and

(2) as a positive control to express the pathogen-specific IFN-γ-response as percent.

A concentration of 0.6 µg/ml of SC resulted in an IFN-γ-reactivity well distributed in the measuring range of the ELISA, whereas lower concentrations often induced weak IFN-γ-production (Fig. 4). To ensure correct interpretation of sample reactivity, a minimum OD_{SC} of 0.4 was defined. Using this validation criterion 93.5% of the samples gave valid results (SC 0.6 µg/ml).

PBS served as negative control and a maximum OD_{PBS} was set to 0.2. Considering both validation criteria SC and PBS 87% of the samples in IFN-γ-profiles were valid. This value is important when the number of samples per herd or group is planned.

5.6.   Unspecific IFN-γ-reactivity to control antigen

A remarkable reactivity against the control antigen (MARC145) was observed (Fig. 5). FCS was identified as the source of this unspecific reactivity. FCS is frequently used as an additive in cell culture. Martelli et al. (2009) and Ferrari et al. (2013) examined 5 to 16 week old pigs (vaccinated and challenged by natural exposure) with an IFN-γ-ELISpot. They resuspended PBMC after purification in an FCS-supplemented medium (10% FCS). As negative control they used cells in the respective medium. They did not use mock-infected cells as a further control antigen for stimulation. None of them reported unspecific reactivity in the negative control. The reason for this might be the age of the animals. In contrast to that in the present studies only sows were tested, which had been repeatedly vaccinated.

Molina et al. (2008) prepared the viral antigen in a medium supplemented with 5% FCS. The PRRSV-antigen was semipurified by a sucrose gradient centrifugation and a medium without supplementation with FCS served as negative control in the IFN-γ-ELISpot. Two week old PRRSV negative piglets were inoculated with PRRSV and bled at 1- to 2-week intervals until the age of 30 weeks. They gained PRRSV-specific IFN-γ-responses, but neither an unspecific reactivity to the control antigen nor the reason for purification of the virus was reported.

Notably two authors reported about unspecific reactivity against the control antigen:

(1) Dotti et al. (2013) used 2% FCS for virus propagation and preparation of the control antigen. The antigens were obtained by freezing and thawing cell cultures, which is similar to the procedure in the current study. The authors observed an IFN-γ-response to the control antigen and speculated about damage-associated molecular pattern molecules causing unspecific reactivity. However, after subtraction of the reactivity against the control antigen most samples scored negative.

(2) Zuckermann et al. (2007) resuspended purified PBMC in medium with 5% FCS. Unstimulated PBMC in this medium served as negative control. No PBS control was carried along. One of their animal groups, treated with a killed vaccine in adjuvant, developed a high frequency of non-PRRSV-specific IFN-γ-producing cells. The IFN-γ-reactivity of negative controls was also subtracted from specific IFN-γ-responses. They assumed the unspecific reactivity to be an effect mediated by a component of the vaccine.

Vaccines, at least the US vaccine used in this study, also contain FCS. It should be kept in mind that other vaccines – vaccines against porcine circo virus 2, porcine parvo virus or swine influenza virus – also may contain FCS. So it might be possible that frequent vaccination leads to unspecific reactivity against the FCS. Therefore, alternative methods for preparation of virus stocks had to be chosen. Purification of virus preparation by means of sucrose gradients or the production of antigen without supplementation of FCS were considered. In this study, the focus was on cell lysates of vaccine virus collected in PBS in order to sustain sufficiently high virus titers. However, titers of field virus after infection of PAM were significantly lower than those of vaccine virus on MARC145-cells. In order to obtain field virus antigens with appropriate titers FCS was substituted by horse serum.

5.7. Feasibility of IFN-γ-RA

So far, the IFN-γ-RA assay was selected as suitable test method and one test kit out of two was chosen for further investigations. The SC was adjusted as reference for IFN-γ-reactivity against pathogen-specific antigens. Unspecific reactivity against control antigen was reduced by an optimisation of antigen preparation. Appropriate

antigens for stimulation of whole blood samples were provided. At this point IFN-γ-RA was ready for feasibility studies.

The aim of a feasibility study is to assess if non-infected animals are identified as negative and infected as positive. This aim requires that non-infected and infected animals are defined as such by a gold-standard. The commercial ELISA might be regarded as such a gold standard. But ELISA-antibodies are developed early after infection and cellular immune response is delayed. This might result in misclassification of animals. To circumvent this problem we defined the status of sow herds. Therefore, blood samples of sows from non-vaccinated/non-infected, non-vaccinated/infected, US- or EU-vaccinated stocks irrespective of infection were studied with IFN-γ-RA. Negative stocks were defined as ELISA-, SNT- and PCR-negative. The absence of IFN-γ-reactivity is expected in these stocks. Additionally, this group was used to estimate the cut-off value for positivity in vaccinated and non-vaccinated/infected stocks. Sows in negative herds tested negative in IFN-γ-RA. Single false-positive animals are no problem because the IFN-γ-RA will not be applied for certification of negative herds. Regarding infected and vaccinated herds a degree of genotype-specificity was observed. US- and EU-IFN-γ-reactivity was preferentially observed in US-vaccinated and EU-vaccinated herds, respectively. Dominance of EU-IFN-γ-reactivity in non-vaccinated/infected herds is in agreement with EU-wild-type infection. Remarkably, a similar pattern was observed for the SNT (Böttcher et al., 2006; Böttcher et al., accepted).

It is the aim of the IFN-γ-RA to detect gaps in immunity. Such gaps might be age-related. Therefore, the available sows were analysed by their litter number as a correlate of age. In US-vaccinated herds a stronger IFN-γ-reactivity was observed against US-antigen. However, sows with litter numbers >4 showed the strongest reactivity and it was directed against both antigens US and EU. This picture might indicate an infection with EU-virus when younger sows were not yet part of the herd. In contrast to that, no age-difference was observed in EU-vaccinated sows. A strong reactivity in non-vaccinated/infected herds in the group 1./2. litter number might be explained by an infection as piglet or gilt of these animals. In summary, IFN-γ-RA provided remarkable differences between animals and herd groups.

5.8.   Comparison of IFN-γ-test with ELISA and SNT

Lopez & Osorio (2004) drawed a picture about the development of viremia, ELISA-antibodies, neutralising antibodies and IFN-γ-response over time. No correlation was observed between ELISA-antibody and IFN-γ-RA. This finding might be due to the fact that IFN-γ-RA differentiated between the EU- and US-genotype, whereas ELISA does not differentiate between genotypes. Additionally, the time difference between the development of ELISA-antibody and cellular immunity might be important. Both development of neutralising antibodies and IFN-γ-responses are retarded after infection, whereas ELISA-antibodies are detected early after infection.

A similar genotype specificity was observed for neutralising antibodies and IFN-γ-RA (Fig. 12, Chapter 4.8.2.). However, IFN-γ-reactivity was not always detected in animals with neutralising antibodies. It should be kept in mind that IFN-γ-reactivity might decrease to undetectable level after successful development of immunity because immune cells are down regulated to a low number of memory cells.

Data of Figure 12 might also indicate a biased immune response. Ideally, a well-balanced immune response should comprise both cellular and humoral immunity. If in a portion of animals only neutralising antibodies but no IFN-γ-response are detected, this could indicate a rather unfavourable immune response. Consequently, IFN-γ-RA might be of remarkable interest in order to detect such an unbalanced immune response.

It should be kept in mind that the feasibility study relied on non-randomly collected blood samples so that obtained data are not representative.

5.9.   IFN-γ-reactivity against field virus isolates

The immune response against field virus isolates is so far an unknown variable which cannot be assessed easily. Lager et al. (1999) examined the humoral immune response in gilts after experimental infection with virulent field virus strains of PRRSV. They compared it to the immune response after vaccination with an attenuated-virus vaccine strain of PRRSV and found a varying development of humoral immunity towards the virus variants. Several PRRSV antigens were included in the present study to examine the differences in cellular immune response against PRRSV isolates. Field virus is currently detected by PCR and was characterized by

sequencing. However, such sequence data are of limited value regarding prediction of antigenicity and possible cross reactivity or cross protection. Since 2006, virus isolation on PAM in routine diagnosis was implemented so that a panel of PRRSV field isolates is available to address immune reactivity to these viruses. In this study four PRRSV field isolates were included for stimulation of blood samples parallely to stimulation with regular antigens. For evaluation of reactivity of blood samples homology of field virus strains to the reference strain Lelystad virus and virus titers of antigens had to be considered. Included field virus strains had a homology ranging from 87% to 93% to reference strain Lelystad. Field virus isolates had lower titers of infectivity (10^4 to 10^6/ml). Therefore, a lower dilution (1:15) in stimulation of blood samples was chosen. Antigens from vaccine virus showed titers of $10^{7.5}$ and $10^{7.8}$/ml and were used at a dilution of 1:150 in stimulation of blood samples. Nevertheless, a remarkable reactivity against field virus strains was observed in EU- and US-vaccinated stocks. These results might indicate a higher power of field virus to induce IFN-γ-responses. So attenuation of virus which is the case for vaccine virus might coincide with reduced power to induce IFN-γ-responses. Field virus isolates are propagated on PAM, cells of the innate immune response, so the cellular background of the antigen might explain a stronger reactivity, too. Reactivity of US-vaccinated sows with field virus-antigens might indicate some cross-protection by US-vaccine. However, this needs to be addressed in further studies. Non-vaccinated/non-infected sows showed a significantly weaker IFN-γ-reactivity than vaccinated/infected sows. Single reactors in non-vaccinated/non-infected herds might pinpoint to such an unspecific reactivity against field virus isolates. However, it should be kept in mind that these herds were characterized by a single testing. So, a history of PRRSV-infection of single animals cannot be ruled out. Mock-infected PAM induced a stronger IFN-γ-reactivity than mock-infected MARC145-controls, but both were subtracted from specific reactivity. Currently, vaccine virus is adapted to PAM in order to compare vaccine virus and field virus isolates by IFN-γ-reactivity.

Díaz et al. (2012) were the first reporting cell-mediated responses against two different PRRSV field strains of the EU-genotype. They isolated the virus strains from infected farms and produced viral stocks in PAM. In two experiments they inoculated pigs with the two EU PRRSV strains and determined IFN-γ-producing cells with the ELISpot after *in vitro* and *in vivo* challenge with the homologous and heterologous virus strain as well. Interestingly, one of the two isolates induced higher frequencies

of IFN-γ-positive cells in both experiments. Ferrari et al. (2013) compared IFN-γ-reactivity in pigs previously vaccinated and subsequently exposed to two field isolates. Heterologous challenge with one field virus isolate induced similar IFN-γ-reactivity compared to homologous challenge with the vaccine virus strain, whereas the other field isolate induced lower IFN-γ-reactivity.

In summary, the presented IFN-γ-RA offers a possibility to include a panel of antigens derived from field virus isolates. It is an important advantage of the IFN-γ-RA compared to the neutralisation assay as field virus isolates are not easily included in neutralisation assays.

5.10.   Stability studies

So far stability of antigens for IFN-γ-RA or IFN-γ-ELISpots was not studied. Therefore, a stability study of antigens was included in order to optimise testing conditions.

Antigens were tested for their stability using different storage conditions. Stimulation capability of specific antigens was not affected by storage conditions. This might be explained by linear epitopes inducing IFN-γ-responses whereas neutralising antibodies are frequently directed against conformational epitopes.

5.11.   Conclusion and future prospects

General feasibility of the IFN-γ-RA has been demonstrated. A provisional cut-off value was defined to differentiate IFN-γ-positive and -negative animals. However, a conclusion about the relevance for protection cannot be drawn. It is still not possible to give a statement on the amount of IFN-γ being protective against PRRSV. IFN-γ-reactivity to specific antigens was rather weak. This result might be explained by the immunobiology of PRRSV-infection.

A strong IFN-γ-reactivity to field virus compared to vaccine virus derived antigens is of remarkable interest. Therefore, the presented IFN-γ-RA is a promising tool to further elucidate the immune response against the field virus. Regarding unspecific IFN-γ-reactivity against mock-infected PAM, further investigations with vaccine derived antigens adapted to PAM are indicated.

Blood samples principally were stimulated with specific antigens at the day of collection. For routine diagnosis this procedure could be a limiting factor. The ability of cells to respond with IFN-γ rapidly decreased. It is a major disadvantage for routine use. However, antigens turned out to be very stable. This is explained by linear epitopes recognised by PBMC.

The present IFN-γ-RA was established with samples collected from sows. The IFN-γ-RA showed a similar genotype specificity as the SNT. The application of IFN-γ-RA on samples from piglets or fatteners remains to be investigated.

A cross-sectional study in sow herds with different vaccination or infection history is currently planned to demonstrate the added value of the IFN-γ-RA. In this study IFN-γ-RA, SNT, ELISA and PCR will be implemented in order to draw a more complete picture of PRRSV-infection.

In summary, the IFN-γ-RA can be used for measuring the cellular immunity. The SNT and the IFN-γ-RA provide two good possibilities to measure appropriate correlates of protective immunity after vaccination against PRRSV.

6.   Summary (English)

Porcine Reproductive and Respiratory Syndrome (PRRS) is a major problem in the swine industry that results in tremendous economic losses. Attenuated live-vaccines, either based on the EU- or the US-genotype, are available for the control of clinical disease. Currently the immune status after vaccination is only controlled by serum neutralisation test (SNT). This study was carried out to establish an IFN-γ-recall assay (IFN-γ-RA) examining the cellular immune response against PRRSV, too.

In the course of routine diagnosis, blood samples were randomly collected from non-vaccinated/non-infected, non-vaccinated/infected and US- or EU-vaccinated herds. A panel of pathogen-specific antigens derived from vaccine virus and field virus isolates was implemented. Appropriate controls (stimulation control (SC), PBS-control and mock-infected cell cultures) were included in order to express results as percent of SC-reactivity and to circumvent an observed unspecific reactivity against cell culture ingredients.

A provisional cut-off (10%) was defined by analysis of non-infected herds. Based on this cut-off value, 28% of sows in US- and 43% of sows in EU-vaccinated herds reacted with US- and EU-antigens, respectively. 29% of sows from non-vaccinated/infected herds preferentially reacted against EU-antigen. Thus the developed IFN-γ-RA showed a similar genotype specificity as observed for the SNT. Field virus derived antigens were included. Compared to vaccine derived antigens, a relatively stronger reactivity in IFN-γ-RA was observed against the field virus derived antigens. This might be due to attenuation of vaccine virus or by the cell culture used for production of antigens. As the field virus was propagated on porcine alveolar macrophages an effect of these cells on results cannot be ruled out.

A remarkable stability of antigens was demonstrated. However, blood samples still need to be stimulated the day of sampling as the ability of cells to respond with IFN-γ rapidly decreased.

Nevertheless, the presented IFN-γ-RA is a promising tool to further analyse the cellular immune response in PRRSV-vaccinated sows.

7.   Summary (German)

Das *Porcine Reproductive and Respiratory Syndrome* (PRRS) ist ein großes Problem in der Schweinehaltung, das zu enormen wirtschaftlichen Verlusten führt. Attenuierte Lebendimpfstoffe, die sowohl auf dem EU- als auch auf dem US-Genotyp basieren, stehen für die Kontrolle klinischer Erkrankungen zur Verfügung. Derzeit wird der Immunstatus nach einer Impfung lediglich mit dem Serumneutralisationstest (SNT) überprüft. In der vorliegenden Studie wurde ein IFN-γ-Test entwickelt, der auch die zelluläre Immunantwort gegen das PRRSV bestimmt.

Im Rahmen von Routineuntersuchungen wurden Blutproben von Sauen aus nicht-geimpften/nicht-infizierten, nicht-geimpften/infizierten und US- oder EU-geimpften Beständen zufällig entnommen. Eine Auswahl an Pathogen-spezifischen Antigenen von Impfvirus- und Feldvirusisolaten wurden in die Untersuchung einbezogen. Geeignete Kontrollen (Stimulationskontrolle (SC), PBS-Kontrolle und nicht-infizierte Zellkulturen) wurden mitgeführt, um die Ergebnisse prozentual zu den IFN-γ-Reaktionen der SC darzustellen und um unspezifische Reaktionen gegen Zellkulturbestandteile zu erfassen.

Ein vorläufiger Cut-off (10%) wurde mit Hilfe nicht-geimpfter/nicht-infizierter Herden festgelegt. Basierend auf diesem Cut-off reagierten in US- und EU-geimpften Beständen 28% der Sauen mit den US- und 43% der Sauen mit den EU-Antigenen. Damit wies der entwickelte IFN-γ-Test eine zum SNT vergleichbare Genotyp-Spezifität auf. Im Vergleich zu den Impfvirus-Antigenen wurden gegen die Feldvirus-Antigene relativ stärkere Reaktionen beobachtet. Die Attenuierung der Impfvirusstämme oder die für die Produktion der Antigene verwendeten Zellkulturen sind mögliche Erklärungen. Da die Feldisolate auf porzinen Alveolarmakrophagen vermehrt wurden, kann ein Einfluss dieser Zellen auf die Ergebnisse nicht ausgeschlossen werden.

Die Haltbarkeit der Antigene war sehr gut. Blutproben müssen jedoch weiterhin am Tag der Entnahme stimuliert werden, da die Fähigkeit der Zellen zur IFN-γ-Produktion mit zunehmendem Abstand von der Entnahme abnimmt.

Dennoch stellt der IFN-γ-Test ein vielversprechendes Werkzeug für weitere Untersuchungen der zellulären Immunantwort in PRRSV-geimpften Beständen dar.

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Annex

Annex 1. Material

Annex 1.1: Cell cultures and antigens

Material	Source	Information
MARC145 cells	Provided from the Collection of Cell lines in Veterinary Medicine (CCLV), Friedrich-Loeffler-Institute (FLI), Riems	Clones of the cell line MA-104 extracted from the fetal kidney of the African Green Monkey
Porcine alveolar macrophages	Extracted in own laboratory	
Ingelvac® PRRS MLV	Boehringer, Ingelheim, Germany	Genotype 2 vaccine virus
Porcilis® PRRS	MSD/Intervet, Boxmeer, Netherland	Genotype 1 vaccine virus
Field isolates	Cultivated in own laboratory	V2276/I, V1192, V683, V995
Control antigens	Prepared in own laboratory	Supernatant of uninfected cell culture
Pokeweed mitogen	Sigma-Aldrich®, St. Louis, USA	Stimulation control

Annex 1.2: Media and reagents

Material	Source	Information
MEM Earle's (Earle's Minimal Essential Medium)	BIOCHROM AG, Berlin, Germany	Basic medium for MARC145, supplemented with 10mM HEPES-Buffer, 1% NEA and 1% Pen/Strep
RPMI 1640 (Roswell Park Memorial Institute Medium)	BIOCHROM AG, Berlin, Germany	Basic medium for PAM, supplemented with 1% NEA, 1% Pen/Strep and 1% L-glutamine
PBS Dulbecco w/o Ca^{2+}, w/o Mg^{2+}	BIOCHROM AG, Berlin, Germany	
PBS Dulbecco (10x)	BIOCHROM AG, Berlin, Germany	
Ultra Pure Water	BIOCHROM AG, Berlin, Germany	
Fetal Bovine Serum/ Fetal Calf Serum gamma-irradiated	Life Technologies™, GIBCO®, Carlsbad, California	
Donor Horse Serum	BIOCHROM AG, Berlin, Germany	
HEPES-Buffer (1M)	BIOCHROM AG, Berlin, Germany	
Non-essential amino acids (100x)	BIOCHROM AG, Berlin, Germany	
Penicillin/streptomycin 10.000 U/ml/ 10.000 µg/ml	BIOCHROM AG, Berlin, Germany	
Gentamycin 10 µg/ml	BIOCHROM AG, Berlin, Germany	
Patricin 50 µg/ml	BIOCHROM AG, Berlin, Germany	
Baytril® 5%	Bayer, Leverkusen, Germany	
L-glutamine 200mM	BIOCHROM AG, Berlin, Germany	

Annex 1.2: Media and reagents (continuation)

Material	Source	Information
Dimethyl sulfoxide	Sigma-Aldrich®, St. Louis, USA	
Tween® 20	Merck Millipore, Darmstadt, Germany	
Bovine serum albumin	Sigma-Aldrich®, St. Louis, USA	
Normal Goat Serum	R&D Systems®, Minneapolis, USA	
CHECKIT* TMB substrate	Idexx Laboratories, Westbrook, USA	Substrate solution
CHECKIT* stop solution TMB	Idexx Laboratories, Westbrook, USA	Stop solution
Ficoll-Paque™ PLUS	GE Healthcare, Buckinghamshire, Great Britain	
Complement sera from guinea pig 776 units/ml	Sigma-Aldrich®, St. Louis, USA	
Trypan blue	Serva Feinbiochemica GmbH, Heidelberg, Germany	
FITC conjugated monoclonal anti-PRRSV antibody	BioX Diagnostics, Jemelle, Belgium	
FITC anti-mouse IgG	Sigma-Aldrich®, St. Louis, USA	
Glycerol	Sigma-Aldrich®, St. Louis, USA	
DABCO	Sigma-Aldrich®, St. Louis, USA	

Annex 1.3: Test kits

Material	Source	Information
DuoSet® ELISA porcine IFN-γ kit	R&D Systems®, Minneapolis, USA	IFN-γ-RA kit A
Porcine IFN-γ-RA kit development	Mabtech, Stockholm, Sweden	IFN-γ-RA kit B
Porcine IFN-γ ELISpot kit	R&D Systems®, Minneapolis, USA	IFN-γ-ELISpot
Herd Check* PRRS X3, Porcine Reproductive and Respiratory Syndrome Virus Antibody Test kit	Idexx Laboratories, Westbrook, USA	Antibody ELISA
QIAamp® Viral RNA Mini-kit	QIAGEN®, Hilden, Germany	Kit includes buffers and 2ml MiniSpin Column collection tube
VIROTYPE® PRRSV	Labor Diagnostik GmbH, Leipzig, Germany	Real time Multiplex RT-PCR Test kit for Detection of EU, NA and HP PRRS viruses

Annex 1.4: Laboratory equipment and supplies

Material	Source	Information
Cell culture vessels	Thermo Scientific, Thermo Fisher Scientific, Waltham, USA	Nunc™ Easy Flasks™, Nunclon™ Delta-treated, 175 v/c, 75 v/c and 25 v/c
96-well microtitration plate	Thermo Scientific, Thermo Fisher Scientific, Waltham, USA	Nunc MicroWell 96-Well Microplates, Nunclon™ Delta Surface, Flat Bottom
48-well multidishes	Thermo Scientific, Thermo Fisher Scientific, Waltham, USA	Nunclon™ Δsurface
Cell tubes	Thermo Scientific, Thermo Fisher Scientific, Waltham, USA	For PAM/MARC145, Nunclon™ Δsurface, flat bottom
High protein binding ELISA plate	Thermo Scientific, Thermo Fisher Scientific, Waltham, USA	Nunc-Immuno™ Plates, MaxiSorp
Micro tubes 1,5ml	Sarstedt, Nürnbrecht, Germany	PP, with attached PP cap
Optical Tube Strips	Agilent Technologies, Santa Clara, California	8x Strip
Stratagene M3005P	Agilent Technologies, Santa Clara, California	
Mx Pro QPCR software	Agilent Technologies, Santa Clara, California	
Cell-Dyn 3500	Abbott, Illinois, USA	
Fuchs Rosenthal counting chamber	Blaubrand®, Thermo Scientific, Thermo Fisher Scientific, Waltham, USA	
Neubauer counting chamber	Blaubrand®, Thermo Scientific, Thermo Fisher Scientific, Waltham, USA	

Annex 1.4: Laboratory equipment and supplies (continuation)

Material	Source	Information
Centrifuge tube	Thermo Scientific, Thermo Fisher Scientific, Waltham, USA	Nunc™, 15ml
Heraeus Megafuge 16R Centrifuge	Thermo Scientific, Thermo Fisher Scientific, Waltham, USA	
Incubator	Memmert, Schwabach, Germany	For cell cultures only 37°C, no CO_2 content
Incubator	Nalge Nunc International, Thermo Fisher Scientific, Waltham, USA	For PAM and field virus propagation 37°C, 0.5% CO_2
Incubator	Nalge Nunc International, Thermo Fisher Scientific, Waltham, USA	For intentions other than cell culture, PAM or field virus propagation, 37°C, 5% CO_2
ELISA reader	TECAN Austria GmbH, Grödig, Austria	Sunrise™
ELISpot reader	AID Autoimmun Diagnostica GmbH, Straßberg, Germany	AID iSpot FluoroSpot Reader System
Intensilight C-HGFI	Nikon, Tokyo, Japan	Fluorescence microscope

Annex 2. IFN-γ ELISA test kits

Annex 2.1: Components of the different IFN-γ-RA's (for origin of components not included in a kit see annex 1)

Testkit		A	B
		Self coating	Self coating
Microplates	Included in testkit	X	X
	Pre-coated	X	X
Wash buffer	Included in testkit	X	X
	Composition	PBS + 0.05% Tween20	PBS + 0.05% Tween20
Reagent diluent/ incubation buffer	Included in testkit	X	X
	Composition	PBS + 1% BSA (Reagent Diluent)	PBS + 0.05% Tween20 +0.1% BSA (incubation buffer)
Capture antibody		Monoclonal mouse anti-porcine IFN-γ, lyophilized	Mouse monoclonal antibody specific for porcine IFN-γ
Standard		Recombinant porcine IFN-γ, lyophilized	Recombinant porcine IFN-γ, lyophilized
Porcine IFN-γ Kit Control		X	X
Detection antibody		Biotinylated polyclonal goat anti-porcine IFN-γ, lyophilized	Biotinylated mouse monoclonal antibody specific for bovine IFN-γ (cross reaction with porcine IFN-γ)
Additional reagents		NSG	X
Streptavidin-HRP		√	√
Substrate Solution		X	X
Stop Solution		X	X

Annex 2.2: Implementation of the different IFN-γ-RA's (according to manufacturer's instructions)

	Kit A	Kit B
The day before implementation	Plate Preparation: Capture Antibody, diluted to 2µg/ml in PBS, 100µl/well, incubation overnight at 4-8°C	Plate Preparation: Capture Antibody, diluted to 2µg/ml in PBS, 100µl/well, incubation overnight at 4-8°C
Day of implementation	Washing: 3 times	Washing: 2 times
	Blocking: 300µl Reagent Diluent/well 1 hour	Blocking: 200µl incubation buffer/well 1 hour
	Washing: 3 times	Washing: 5 times
	Standard and sample, diluted 1:2 in Reagent Diluent, 100µl/well 2 hours	Standard and sample, diluted 1:2 in incubation buffer, 100µl/well 2 hours
	Washing: 3 times	Washing: 5 times
	Detection Antibody, diluted to 400ng/ml in Reagent Diluent with 2% heat inactivated normal goat serum (NGS), 100µl/well 2 hours	Detection antibody, diluted to 500ng/ml in incubation buffer 100µl/well 1 hour
	Washing: 3 times	Washing: 5 times
	Streptavidin-HRP, diluted 1:200, 100µl/well 20 minutes	Streptavidin-HRP, Diluted 1:1000, 100µl/well 1 hour
	Washing: 3 times	Washing: 5 times
	Substrate Solution, 100µl/well 20 minutes	Substrate Solution, 100µl/well 10 minutes
	Stop Solution, 100µl/well	Stop Solution, 100µl/well

Annex 3. Recipes for preparation of buffers

Annex 3.1: Recipe for 1l of PBS + 0.05% Tween 20 (wash buffer, Kit A and B)

100ml   PBS (10x)

900ml   aqua distillata (aqua dist.)

500µl   Tween® 20 (Merck Millipore)

Annex 3.2: Recipe for 1l of PBS + 1% BSA (Reagent Diluent, Kit A)

100ml   PBS (10x)

900ml   aqua distillata (aqua dist.)

10g   bovine serum albumin (BSA, Sigma-Aldrich®)

Annex 3.3: Recipe for 1l of PBS + 0.05% Tween 20 + 0.1% BSA (incubation buffer,
               Kit B)

100ml   PBS (10x)

900ml   aqua distillata (aqua dist.)

500µl   Tween® 20 (Merck Millipore)

1g   bovine serum albumin (BSA, Sigma-Aldrich®)

Annex 4. IFN-γ-reactivity in stocks and evaluability of samples: Exclusion of samples from analysis by application of validation criteria.

Status of stocks	Samples (total)	SC<0.4	PBS≥0.2	Percent of evaluable samples
		OD		
Non-vaccinated/ non-infected	9	-	-	100%
	9	2	-	78%
	10	-	-	100%
Non-vaccinated/ infected	9	-	4	56%
	10	2	-	80%
	11	-	-	100%
US-vaccinated	11	-	-	100%
	10	3	-	70%
	10	1	-	90%
	10	-	4	60%
	10	2	1	70%
	10	-	1	90%
	10	1	-	90%
	10	3	-	70%
	9	-	-	100%
	10	-	-	100%
EU-vaccinated	10	1	2	70%
	10	-	-	100%
	10	-	-	100%
	10	-	2	80%
	5	-	-	100%
	10	-	-	100%
	10	-	1	90%
	10	1	1	80%
	10	-	-	100%
	10	-	-	100%
	10	-	1	90%
	10	1	-	90%
	7	-	-	100%
	10	3	-	70%
	10	-	-	100%
	300	20	17	87,9%

A5: Correlation of results between antibody ELISA, SNT and IFN-γ ELISA.

ELISA	SNT	IFN-γ	% of total samples
Positive (71.3%)	Negative (9%)	Negative	6.1
		US positive	0.4
		EU positive	2.1
		US/EU positive	0.4
	US positive (5.7%)	Negative	4.9
		US positive	0
		EU positive	0
		US/EU positive	0.8
	EU positive (38.9%)	Negative	24.6
		US positive	0
		EU positive	13.1
		US/EU positive	1.2
	US/EU positive (17.7%)	Negative	7
		US positive	0.4
		EU positive	7.8
		US/EU positive	2.5
Negative (28,7%)	Negative (20.1%)	Negative	16.8
		US positive	2.5
		EU positive	0.4
		US/EU positive	0.4
	US positive (2.8%)	Negative	1.2
		US positive	1.6
		EU positive	0
		US/EU positive	0
	EU positive (3.7%)	Negative	2.5
		US positive	0.4
		EU positive	0
		US/EU positive	0.8
	US/EU positive (2.1%)	Negative	2.1
		US positive	0
		EU positive	0
		US/EU positive	0

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