Research paper

PRRSV RNA detection in different matrices under typical storage conditions in the UK

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In the UK, approximately 40% of the pig breeding herds are outdoors. To monitor their porcine reproductive and respiratory syndrome virus (PRRSV) status, blood is collected commonly from piglets around weaning. Sample collection in British outdoor pigs often occurs during the early morning hours when the piglets tend to accumulate inside sheltered areas. For practical reasons, dry cotton swabs are occasionally used for blood collection and stored at room temperature until arrival in the laboratory. Detection of PRRSV RNA is a function of viral concentration, sample type, and storage condition. To evaluate a possible impact of the sampling protocol on PRRSV species 1 (PRRSV1) detection, experimentally spiked blood samples using three dilutions of a representative PRRSV1 strain were prepared. In addition, blood samples from pigs naturally infected with PRRSV were obtained from a PRRSV-positive British herd. Spiked blood and blood from infected pigs were used to obtain sera, dry or wet (immersed in saline) polyester or cotton swabs, and FTA® cards. The different samples were stored for 24h, 48h, or 7d at 4°C or 20°C and tested by a real-time reverse transcriptase PRRSV PCR assay. Under the study conditions, the best matrix was serum (96.7%), followed by wet swabs (78%), dry swabs (61.3%), and FTA® cards (51%). Polyester swabs (76%) showed a better performance than cotton swabs (63.3%). The reduction in sensitivity obtained for swabs and FTA® cards was particularly high at low viral concentrations. The results indicate that wet polyester swabs should be used whenever possible.

Keywords: PRRSV species 1; detection; swabs, FTA® card; real-time RT-PCR assay.

Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is one of the most important pig pathogens impacting the health of pigs across all age groups.[1] Infection of breeding herds can result in reduced performance due to abortions, increases in mummy and stillbirth rates, and prolonged returns to service. In growing pigs, PRRSV infection signs include respiratory disease, decreased growth rates, increased mortality, and an increase in secondary infections, often resulting in an increase in the use of antimicrobials. PRRSV was identified almost simultaneously in North America and Europe between 1980–1990.[2] Estimated median annual cost of PRRSV for European farms ranged from €75,724 when the farm was slightly affected to €650,090 when the farm was severely affected.[3] Based on the genomic sequences, PRRSV isolates can be divided into species 1 (PRRSV-1) and 2 (PRRSV-2).[4,5]

In British pigs, only PRRSV-1 is present[6] but despite the availability of commercial vaccines, PRRSV continues to be a problem for many pig producers. Determining the PRRSV status of a herd is critical to understand the disease dynamics and to design effective control or elimination. Guidelines to reach a PRRSV free herd status have been established by the American Association of Swine Veterinarians (AASV).[1] Testing end of nursery and mid finishing pigs is recommended and generally, the number of pigs sampled will be determined by the expected prevalence, required confidence intervals, test specifics, as well as farm specifics such as pig flow.[1] Nucleic acid and antibody detection are the most common laboratory tests used for the diagnosis of PRRSV. For many years, serum collected from individual pigs was considered the best diagnostic sample type for PRRSV monitoring and surveillance. However, other matrices

such as oral fluid and blood swabs have been repeatedly reported to be similarly reliable for the diagnosis of PRRSV.[7–9]

Blood sampling is a veterinary act in the UK and veterinary services are not routinely provided out-with normal working hours. In outdoor herds, blood is commonly collected at weaning via ear vein puncture onto either cotton or polyester swabs, with the aid of supervised trained lay people. The swabs are placed into tubes containing liquids such as saline (wet) or into empty tubes or the paper sleeve they came in (dry).

Blood swabs in particular have been used for the diagnosis of PRRSV-1 and PRRV-2 with detection rates similar to those obtained in serum.[8, 10–13] Both cotton and polyester swabs have been suggested for routine diagnostic investigations[14, 15] although the reliability of cotton swabs for virus recovery has not been evaluated in blood samples. Some studies have reported a reduced level of nucleic acid detection in polyester blood swabs when compared with serum, which was attributed to the inherent dilution effect produced when swabs are immersed in saline solution.[9] The use of FTA® cards or dry swabs could be an alternative to wet swabs. FTA® cards potentially could facilitate sample collection greatly since the only materials needed are the FTA® cards and needles, and the cards can be transported at room temperature.

The objective of this study was to determine the accuracy of PRRSV RNA detection for sample types typically used in the UK spiked with a PRRSV-1 isolate under experimental conditions and to compare those results with the same collection materials and storage conditions using samples from naturally infected animals.

Materials and methods

Ethical statement

The PRRSV negative blood samples used in this study were collected as part of routine health surveillance program of a British pig herd. The samples from pigs naturally infected with PRRSV were collected during a diagnostic investigation of a known PRRS positive breeding herd (Fig. 1).

Sample processing

To generate serum and blood samples with a defined PRRSV concentration, 10-fold serial dilutions of PRRSV-1 strain H2 (Genbank accession number AF378799.1) stock with a virus titre of 0.4×10^6 50% tissue culture infectious dose ($TCID_{50}$) per ml were prepared. The experiment was performed using five replicates and three 10-fold serial dilutions including 0.4×10^3 $TCID_{50}$/ml (high), 0.4×10^2 (medium) and 0.4×10^1 (low) $TCID_{50}$/ml. Six different sample types were used including serum, blood swabs using polyester (Telirene) tip swabs (TS19-G, Technical Service Consultants Ltd) or cotton tip swabs (TS8-A, Technical Service Consultants Ltd) and blood collected on FTA® cards (Whatman®, GE Healthcare Life Sciences) (Fig. 2). The swabs were dipped in each of the blood dilutions until the saturation point was reached and immediately placed into a tube containing 1 ml of saline (wet swabs) or allowed to dry at room temperature (20°C) for 16h (dry swabs). FTA® cards were prepared by adding 0.15 ml of each blood dilution and were dried uncovered at room temperature for 16h. Likewise, five PRRSV RNA positive blood samples from naturally PRRSV infected animals were selected to prepare the serum, swabs and FTA® cards.

Sample storage

Samples were stored until processing as described in Table 1 and Table 2. Overall, 285/515 samples including wet polyester swabs, dry polyester swabs, wet cotton swabs, dry cotton swabs, and serum were kept at 4°C to simulate posting with ice packs and the remaining 230 samples were kept at room temperature. All 60 FTA® cards were kept at room temperature for the duration of the experiment following the manufacturer's instructions. At each storage time of 24h, 72h, or 7 days, five samples of naturally infected animals or five samples per sample type for each of the three viral dilutions were obtained for nucleic acid extraction. These times and conditions were chosen to mimic shipment from the farm to the laboratory. Even in the event of delays, samples are expected to arrive 24-72h after shipment.

RNA extraction

Prior to testing, the dry swabs were rehydrated for 30 min by placing them in a tube containing 1 ml of saline. From each FTA® card, 3 × 2 mm diameter punches were eluted in 0.1 ml of RNA rapid extraction solution (AM9775, Thermo Fisher Scientific) for 5 min. For a subset of samples, an additional single 6 mm diameter FTA® card punch was eluted and tested by RT-PCR. As the PRRSV RNA detection limit was similar for both punch methods, 3 × 2 mm punches were chosen and used throughout the study herein. Viral RNA extraction was carried out with a KingFisher Flex 96-tip comb from Thermo Scientific using a MagMAX-96 viral RNA isolation kit (AM1836, Thermo Fisher Scientific) according to the manufacturer's instructions.

Detection of PRRSV RNA

The nucleic acids were tested by a commercial real-time RT-PCR kit (TaqMan NA and EU PRRSV Reagents, Thermo Fisher Scientific) according to the manufacturer's instructions on an

ABI 7500 thermocycler. A cycle threshold (Ct) value > 37 was considered negative. Positive and negative controls were included in each run. Genomic equivalent titers of PRRSV were determined based on serial dilutions of a commercial positive control (VetMAX™ NA and EU PRRSV and Xeno™ RNA Controls, Thermo Fisher Scientific).

Statistical analysis

PRRSV genomic copy equivalents were log transformed prior to analysis by using linear mixed models fitting the effects of storage temperature, storage time, sample type, and PRRSV titre as fixed effects while the replicate was the random effect. Within main effects, significance of differences between individual treatment means was determined by using Tukey's honestly significant difference test. Differences in detection ratios of positive and negative for PRRSV RNA were analysed by Fisher's exact test. Statistical significance was set at $p < 0.05$. Data analysis was done with SAS 9.3 (SAS Institute, Cary, NC, USA).

Results

Detection of PRRSV RNA in samples with controlled laboratory PRRSV contamination

Detection of PRRSV positive dilutions for the different sample types are summarised in Table 1. In experimentally contaminated samples, sample type, temperature, time of storage and PRRSV titre had a significant effect in PRRSV RNA levels ($p < 0.001$ for all variables, Supplemental Table 1). Overall, the best sample type was serum (PRRSV RNA copies 2.63±0.04), followed by polyester swabs (1.47±0.04), cotton swabs (1.22±0.04), and FTA® cards (0.98±0.05). PRRSV RNA was detected in 96.7% (87/90), 76% (114/150), 63.3% (95/150) and 51% (23/45) of the

142 serum, polyester swabs, cotton swab and FTA® card samples. When combining data from
143 polyester and cotton swabs, wet swabs had a higher PRRSV RNA detection rates and load (78%,
144 117/150; 1.62±0.05) than dry swabs (61.3%, 92/150; 1.07±0.05) ($p < 0.0001$, Supplemental Table
145 1). There was no interaction between swab material type (polyester or cotton) and storage medium
146 (dry or wet) ($p = 0.47$).
147 **Serum versus swabs.** Considering the conditions tested for both serum and swabs (Table 1),
148 PRRSV RNA detection rate was higher in serum (98.6%, 74/75) than swabs (69.6%, 209/300) (p
149 < 0.001). Comparing the performance of the swab tip material, the detection rate was higher for
150 polyester (76%, 114/150) than cotton (63.3%, 95/150) ($p < 0.001$). Comparing the PRRSV RNA
151 recovery rates when blood swabs were immediately placed in saline after collection or kept dry
152 until processing, detection rates were higher for wet swabs (78%, 117/150) than dry swabs
153 (61.3%, 92/150) ($p = 0.01$).
154 When swab types were further divided, the highest PRRSV RNA detection rates were obtained for
155 wet polyester (82.6%, 62/75), followed by wet cotton (73.3%, 55/75), dry polyester (69.3%,
156 52/75), and dry cotton (53.3%, 40/75). The virus genome detection rate in wet polyester was still
157 lower than in serum samples ($p < 0.001$). Differences in detection rates between swabs and serum
158 were mainly due to the low detection rates in swabs spiked with the lowest virus dilution
159 ($p<0.001$).
160 **Dry versus wet swabs.** Wet swabs had higher detection rates than dry swabs at 4°C ($p = 0.03$) and
161 20°C ($p < 0.01$). The PRRSV RNA detection rates in wet swabs were higher than dry swabs after
162 24 h ($p = 0.02$) and 72 h of storage ($p < 0.001$) but not after 7 d of storage ($p = 0.08$). A higher

number of wet swabs were positive for PRRSV RNA compared to dry swabs for samples spiked with medium ($p < 0.001$) and low ($p < 0.01$) virus titres. There was no effect of storage time or temperature on the virus genome detection rates within the same swab type, although detection was numerically higher for shorter storage periods at lower temperature. On the contrary, viral titre had a significant effect on the detection of PRRSV within the same swab type. The positive detection rate was significantly lower for samples spiked with low titres compared to medium and high for both wet ($p < 0.001$) and dry swabs ($p < 0.05$).

Serum versus FTA® cards. When serum and FTA® cards were compared, serum had a positive detection rate of 95.6% (43/45) while FTA® cards detected 48.9% (23/45) samples as positive ($p < 0.001$). This was due to low detection rate in the blood FTA® cards spiked with moderate ($p = 0.003$) and low ($p < 0.001$) virus titres.

FTA® cards versus swabs. The detection ratio of 56.6% (17/30) obtained with the FTA® cards was lower than the 83.3% (25/30, $p<0.001$) and 73.3% (22/30, $p = 0.02$) obtained with wet polyester and cotton swabs. Both types of dry swabs detected a similar number of positive samples compared to the FTA® cards.

Detection of PRRSV RNA in samples from naturally infected pigs

Detection of PRRSV RNA in samples from naturally infected animals for the different matrices and storage conditions is summarised in Table 2 and Supplemental Table 1. The detection rates and PRRSV RNA load in naturally infected pigs (26/100) was similar to the samples spiked with a low virus amount (19/100) ($p = 0.30$). Wet polyester swabs had higher detection rates (40%, 12/30) than dry polyester swabs (20%, 6/30), wet cotton swabs (20%, 6/30), dry cotton swabs

(0%, 0/30), and FTA® cards (0%, 0/30), however there was no difference in mean PRRSV RNA load among those sample types (Supplemental Table 1). PRRSV RNA detection rates in dry cotton swabs and FTA® cards were lower when compared to wet polyester ($p < 0.001$), wet cotton ($p = 0.01$) and dry polyester ($p = 0.011$). Although wet polyester had the highest PRRSV detection rates ($p = 0.02$) and PRRSV RNA loads ($p < 0.001$) among swabs, the highest detection rates among all sample types were for serum samples ($p = 0.02$).

Discussion

In this work, the efficacy of detecting PRRSV RNA in typical samples types used in the UK was investigated using a standard extraction and RT-PCR protocol. The highest PRRSV RNA detection rates were obtained using serum samples compared to the other sample types that used blood as specimen. Differences between detection ratios in blood samples and serum could be related to RT-PCR inhibitors such as haemoglobin and EDTA[16] as EDTA was used to prepare the blood swabs and FTA® cards. In a previous study, detection of PRRSV RNA in fresh blood swabs from naturally infected boars was similar to the detection in serum.[8]

Wet polyester swabs had the highest PRRSV RNA detection rates (82.6%) among swab types suggesting that the virus release efficiency in this sample type was superior to wet cotton swabs (73.3%), dry polyester swabs (69.3%) and dry cotton swabs (53.3%). Polyester swabs have been recommended for RNA viruses detection when compared to cotton swabs due to inhibitory factors present in treated cotton.[16] When wet and dry swabs were compared at the same storage time and temperature, the number of PRRSV RNA copies was lower for dry swabs even when

detection rates were similar. A previous study investigating the detection of African swine fever virus DNA in serum or dry blood swabs of experimentally infected pigs found no difference in the detection rates between these two sample types after storage for eight days at room temperature.[17] Differences between studies may be due to the different viral load in the original samples, virus structure (RNA versus DNA) and swab type used. In this study, the lowest PRRSV RNA detection was in FTA® cards (48.9%). Many studies have reported decreased sensitivity of RT-PCR for RNA viruses spiked *in vitro* on FTA® cards.[11, 12, 18] This loss of sensitivity has been estimated to be about 100 times lower than in the native sample material.[12] The lower the viral titer of the spiked samples, the less favourable non-serum sample types performed with regards to detection rate and PRRSV RNA copy numbers detected. Spiking samples with cell culture propagated PRRSV may not reflect samples from naturally infected pigs as PRRSV is a mostly cell-associated virus, although non-cell-associated virus has been reported in serum of infected boars.[19] In here the detection rates and mean PRRSV RNA copies in samples from pigs infected with PRRSV naturally were similar to the detection rates of samples spiked with the lowest virus amount. When expecting low amounts of the virus to be circulating in the animals to be sampled, as for example in herds infected with low virulent PRRSV[13], this finding may be important and could direct the farm towards a different sampling protocol.

In this study, only individual samples were tested. However, it is common to test pooled samples to reduce the overall costs of the RT-PCR testing and it has been demonstrated that when pooling samples, the detection levels can be reduced if low viral titres are present.[8] Based on the

detection rates and PRRSV genomic load in samples from naturally infected animals in this study, pools of three or more swabs would likely be negative.

In conclusion, the overall best sample type was serum followed by wet polyester swabs, while dry cotton swabs and FTA® cards had the lowest detection rates when samples contained low amount of virus.

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Competing interests None declared.

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Table 1. Detection of PRRSV RNA in different sample types (serum or blood collected via wet or dry polyester or cotton swabs or FTA® cards) at different storage temperatures (4°C or 20°C) and times (24 h, 72 h or 7 d) prior to RNA extraction. Blood samples were spiked with PRRSV isolate H2 at a titre of 0.4×10^3 to 0.4×10^1 $TCID_{50}$/ml. Data are presented as number of positive samples/total number of samples (mean log PRRSV RNA copy numbers ± SD). A sample with a cycle threshold (ct) equal or greater than 37 was considered negative.

Storage		**Viral titre**	**Serum**	**Wet swab**		**Dry swab**		**FTA® card**
Temperature	*Time*	**($TCID_{50}$/ml)**		*Polyester*	*Cotton*	*Polyester*	*Cotton*	**3 × 2 mm punches**
4°C	**24 h**	1×10^3	5/5 (3.7±0.2)^{A,1}	5/5 (2.9±0.2)^{B}	5/5 (2.6±0.3)^{B}	5/5 (2.5±0.4)^{B}	5/5 (2.3±0.2)^{B}	Not done
		1×10^2	5/5 (2.8±0.3)^{A}	5/5 (2.0±0.2)^{B}	5/5 (1.9±0.3)^{B}	5/5 (1.5±0.6)^{BC}	5/5 (1.3±0.1)^{C}	Not done
		1×10^1	4/5 (1.5±0.9)^{A}	3/5 (0.7±0.7)^{B}	0/5 (0)^{B}	1/5 (0.2±0.5)^{B}	0/5 (0)^{B}	Not done
	72 h	1×10^3	5/5 (3.9±0.1)^{A}	5/5 (3.1±0.0)^{B}	5/5 (3.0±0.1)^{B}	5/5 (2.8±0.1)^{BC}	5/5 (2.4±0.1)^{C}	Not done
		1×10^2	5/5 (2.9±0.1)^{A}	5/5 (2.1±0.1)^{B}	5/5 (2.0±0.2)^{B}	5/5 (1.8±0.2)^{B}	4/5 (1.0±0.6)^{C}	Not done
		1×10^1	5/5 (1.9±0.2)^{A}	3/5 (0.5±0.4)^{B}	2/5 (0.4±0.6)^{B}	2/5 (0.3±0.5)^{B}	0/5 (0)^{B}	Not done
	7 d	1×10^3	5/5 (3.7±0.1)^{A}	5/5 (2.7±0.0)^{B}	5/5 (2.6±0.1)^{B}	5/5 (2.5±0.1)^{BC}	5/5 (1.9±0.2)^{C}	Not done
		1×10^2	5/5 (2.6±0.2)^{A}	5/5 (1.6±0.3)^{B}	5/5 (1.6±0.2)^{B}	5/5 (1.5±0.3)^{B}	0/5 (0)^{C}	Not done
		1×10^1	5/5 (1.7±0.2)^{A}	1/5 (0.2±0.6)^{B}	1/5 (0.8±0.4)^{B}	1/5 (0.2±0.5)^{B}	0/5 (0)^{B}	Not done
20°C	**24 h**	1×10^3	5/5 (3.5±0.2)^{A}	5/5 (2.7±0.3)^{B}	5/5 (2.2±0.6)^{B}	5/5 (2.0±0.3)^{B}	5/5 (2.2±0.4)^{B}	5/5 (2.1±0.1)^{B}
		1×10^2	5/5 (2.6±0.1)^{A}	5/5 (2.0±0.2)^{AB}	5/5 (1.8±0.3)^{B}	1/5 (0.2±0.5)^{C}	2/5 (0.7±0.9)^{C}	3/5 (0.8±0.7)^{C}
		1×10^1	5/5 (1.9± 0.1)^{A}	2/5 (0.5±0.6)^{B}	0/5 (0)^{B}	0/5 (0)^{B}	0/5 (0)^{B}	0/5 (0)^{B}
	72 h	1×10^3	5/5 (4.0±0.1)^{A}	5/5 (3.1±0.1)^{B}	5/5 (3.0±0.1)^{B}	5/5 (2.7±0.1)^{BC}	5/5 (2.3±0.1)^{C}	5/5 (2.0±0.1)^{C}
		1×10^2	5/5 (3.0±0.1)^{A}	5/5 (2.1±0.1)^{B}	5/5 (1.9±0.1)^{B}	5/5 (1.7±0.2)^{B}	4/5 (0.8±0.5)^{C}	4/5 (0.9±0.5)^{C}
		1×10^1	5/5 (2.0±0.2)^{A}	3/5 (0.7±0.6)^{B}	2/5 (0.4±0.6)^{B}	2/5 (0.3±0.5)^{B}	0/5 (0)^{B}	0/5 (0)^{B}
	7 d	1×10^3	5/5 (3.0±0.2)^{A}	Not done	Not done	Not done	Not done	5/5 (2.1±0.2)^{B}
		1×10^2	5/5 (2.1±0.2)^{A}	Not done	Not done	Not done	Not done	1/5 (0.2±0.3)^{B}
		1×10^1	3/5 (0.6±0.5)^{A}	Not done	Not done	Not done	Not done	0/5 (0)^{A}

[1] Different superscripts (^{A,B,C}) within a row indicate significant differences in mean PRRSV genomic copies for a sample type.

Table 2. Detection of PRRSV RNA in different sample types (serum, blood collected via moist or dry polyester or cotton swabs or FTA® cards) at different storage temperatures (20°C or 4°C) and times (24 h, 72 h or 7 d) prior to RNA extraction. Blood samples are from naturally infected pigs. Data are presented as number of positive samples/total number of samples (mean log PRRSV RNA copy numbers ± SD). A sample with a cycle threshold (ct) equal or greater than 37 was considered negative.

Storage		**Serum**	**Wet**		**Dry**		**FTA® card**
Temperature	*Time*		*Polyester*	*Cotton*	*Polyester*	*Cotton*	**3 × 2 mm punches**
4°C	**24 h**	Not done	2/5 (0.46±0.64)	1/5 (0.21±0.47)	1/5 (0.21±0.48)	0/5 (0)	Not done
	72 h	Not done	2/5 (0.50±0.71)	0/5 (0)	1/5 (0.19±0.42)	0/5 (0)	Not done
	7 d	Not done	2/5 (0.46±0.64)	1/5 (0.22±0.49)	0/5 (0)	0/5 (0)	Not done
20°C	**24 h**	Not done	3/5 (0.73±0.72)	1/5 (0.20±0.46)	2/5 (0.42±0.58)	0/5 (0)	0/5 (0)
	72 h	Not done	2/5 (0.41±0.56)	3/5 (0.59±0.54)	1/5 (0.17±0.39)	0/5 (0)	0/5 (0)
	7 d	5/5 (1.62±0.42) [A]	1/5 (0.26±0.59)[B]	0/5 (0) [B]	1/5 (0.23±0.52) [B]	0/5 (0) [B]	0/5 (0) [B]

[1] Different superscripts ([A,B]) within a row indicate significant differences in mean PRRSV genomic copies for a sample type.