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Paper

Comparative effectiveness of different biological specimens for PRRSv detection in naturally infected fattening pigs

Milena Monnier[1], Silvia Marro[2], Andrea Trossi[1*], Marco Faccenda[3], Nicoletta Vitale[1], Roberto Zoccola[1], Ilaria Miceli[1], Francesca Rossi[1], Simone Peletto[1], Alessandro Dondo[1], Mariella Goria[1]

[1]*Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta - IT*
[2]*ASL TO5, Local Health Unit of Chieri, Carmagnola, Moncalieri and Nichelino - IT*
[3]*Professional Veterinarian - IT*

[*]*Corresponding author at: Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta - IT*
E-mail: andrea.trossi@izsplv.it

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Abstract

One of the most critical aspects of PRRS outbreak control in swine herds is the reliable virus detection in both newly introduced animals and replacement gilts. In present study we compared the effectiveness of different biological specimens which - alone or in combination - allow to detect PRRSv carrier animals by mean of Reverse Transcriptase nested PCR (RT-nPCR). Five different matrices (serum, nasal swab, oral fluid, tracheobronchial swab and bronchoalveolar lavage fluid - BALF), known to be effective for PRRSv detection, were included in diagnostic efficacy comparison. Thirty-five pigs newly introduced into a fattening unit of a PRRS chronically affected farm were randomly selected to be submitted to serial sampling of each of the matrices above described, during the first three months of fattening period. A Bayesian approach was used to analyze the RT-nPCR results (i.e., positive or negative) of each sampling method and their respective sensitivity was estimated. PRRSv was detected by RT-nPCR in at least one matrix from 58% to 100% of the pigs. Tracheobronchial swabbing, as well as the combination of tracheobronchial swabbing plus bronchoalveolar washing, or tracheobronchial swabbing plus serum sampling were proved to be the most sensitive sampling methods to detect PRRSv in naturally infected live pigs. This study enlightens as the tracheobronchial swabbing associated with RT-nPCR could be the most recommended diagnostic tool for assessing infection dynamics in pig herds.

Keywords

PRRSv, Serum, Swabs, Oral fluid, BALF, RT-nPCR

Introduction

The etiological agent of Porcine Reproductive and Respiratory Syndrome (PRRS), PRRS Virus (PRRSv), was first identified in 1991 (Wensvoort et al., 1991) and has since been recognized globally as one of the most economically important infectious agents affecting swine. PRRSv causes reproductive failure in sows and interstitial pneumonia in growing and finishing animals (Halbur et al., 1996; Rossow et al., 1996).

PRRSv replicates predominantly in the alveolar macrophages (PAMs) of the lung; it can induce prolonged viremia and causes persistent infections that last for months after the onset of the disease (Wensvoort et al., 1991). PRRS is one of the most challenging diseases affecting the pig industry due to its high mutation rate and genetic variability, even among viruses within the same genotype, which leads to the lack of cross-protection against heterologous strains and limits the efficacy of currently available vaccines (Mateu et al., 2007). Disease prevention is based on a combination of vaccination and management strategies (i.e., control of animal flow, acclimatization of gilts, and adoption of internal and external biosecurity measures) (Rowland et al., 2017). Therefore, it is important to prevent the introduction of new viral strains from outside and reduce the risk of mutations by limiting viral circulation, promoting the establishment of protective immunity against the field strain, and maintaining the status of negativity in a free herd.

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Surveillance of the disease is a key component in the prevention and/or control of losses from clinical cases. PRRSv infection may result in clinically normal, but persistently infected animals. In this context, the most critical step is to identify the carrier status of animals newly introduced into the herd. The detection of infected animals is important to control PRRS and to reduce the potentially serious economic damage that can result from an outbreak.

Reverse Transcriptase Nested PCR (RT-nPCR) is the most commonly used diagnostic assay for monitoring PRRSv infection in swine herds. In farms where the infection has been eradicated and no clinical signs of PRRS are detectable, this diagnostic test can be used to determine whether viral circulation is still present (Duinhof et al., 2011). The matrix most frequently used for the diagnosis of PRRS is serum, which allows efficient and cost-effective sampling since it can be used to detect both the antibodies and the virus. RT-nPCR on serum highlights the early phase of viremic infection, but frequently can alter the diagnosis (Duinhof et al., 2011). Horter et al. first indicated that other specimens, such as oral fluid, tracheobronchial swab, nasal swab, and bronchoalveolar lavage fluid (BALF), could be used as alternatives to detect PRRSv (Horter et al., 2002). Currently, different specimens have been tested for the detection of PRRSv. The low cost and availability of optimized tools for oral fluid sampling made it possible to take advantage of this specimen in the veterinary field (Chittick et al., 2011) and in PRRSv detection (Plut J. et al., 2020). In particular, one of the main reasons for choosing oral fluid is its stress-free ease of use. The objective of this study was to determine which specimen is most effective in identifying infected animals in a PRRS-infected fattening pig unit.

Materials and methods

Experimental design

The diagnostic performances of five different biological specimens (serum, nasal swab, oral fluid, tracheobronchial swab, and BALF) were evaluated for the diagnosis of PRRSv. Thirty-five pigs were monitored in a commercial swine herd known to be PRRSv-infected. This farm houses up to 4,000 pigs per cycle and consists of six sheds. When introduced into the sheds, piglets were 10-11 weeks old, weighing about 25 kg, and they remained there until reaching slaughter age. Subjects involved in this study did not undergo experimental infection with PRRSv but were naturally exposed to the virus on the farm, creating a more realistic context: in commercial farms, breeders would not typically aim to trigger an experimental infection, considering the large amount of virus required and the relevant regulatory limits for animal testing. Nevertheless, viral circulation of PRRS was confirmed by the herd's anamnestic history, the positivity of the mothers of selected subjects, and the viremia detected by RT-nPCR on serum in the pig group the day before they were moved to the sheds. The sample size was defined to achieve a lower limit of the 95% CI equal to 75%, for an expected diagnosis of 95%, with an error margin of ± 5%. Piglets were grouped into seven boxes, and sampling began when the animals were 12 weeks old, with specimen collection every two weeks for three months.

Sample collection

Each animal, before sampling, was restrained with a rigid rod jaw clamp. Samples of serum, bronchoalveolar lavage fluid, oral fluid, nasal secretion, and bronchial swabs were collected six times for analysis by RT-nPCR. Blood was drawn from the jugular vein into vacutainer tubes. Nasal secretions were collected by gently scraping swabs along the walls of the nasal cavity, while bronchial secretions were obtained using a disposable probe. Bronchoalveolar lavage was performed following the technique described by Scollo et al. (2011), accessing the lungs orally with a disposable probe approximately 90 cm long. A total of 30 ml of sterile physiological solution was introduced, and 2 ml of the washing fluid was recovered. Finally, oral fluid was aspirated using a disposable syringe (without a needle) from the buccal vestibule and sublingual floor.syringe (without a needle) from the buccal vestibule and sublingual floor.

Nucleic Acid Extractions

Total RNA was extracted from serum, bronchoalveolar lavage fluid, nasal swabs, and bronchial swabs using the E.Z.N.A. Viral RNA Kit (Omega Bio-tek, Inc., USA) and the QIAamp Viral RNA Mini Kit (Qiagen, Germany), following the manufacturer's instructions, based on their laboratory availability. Both RNA extraction kits yielded excellent and comparable results in an interlaboratory test for PRRSv molecular detection organized by the Istituto Zooprofilattico Sperimentale delle Venezie.

Oral fluid specimens were handled by adding RNA-later (Qiagen) to the sample matrix immediately after collection, followed by refrigeration. The RNeasy Micro Kit (Qiagen, Germany) was then used for RNA extraction. Additionally, a

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set of samples was collected without RNA stabilizer, using the same cooling conditions. RNA extraction was performed using the semi-automatic KingFisher Extractor (Thermo Fisher Scientific Inc., USA) with the MagVet™ Universal Purification Kit, according to the manufacturer's protocol.

Nested PCR

A previously published RT-nPCR assay was selected to assess viral presence in the samples (Persia et al., 2001). This assay uses specific primers targeting the ORF7 sequence at the 3' end of the PRRSv genome for both the European (EU) and North American (NA) strains (Table 1).

To enhance the sensitivity of the method for detecting the European strain, two additional nested primers were designed and used in a second amplification step. The preparation for this second PCR step was as follows: 32.6 µl DEPC water, 5 µl of 10x PCR buffer, 1 µl of 10 mM dNTP mix, 1.7 µl of PRRS INT I and PRRS INT II (20 µM), 2 µl MgSO4, 1 µl Taq DNA Polymerase Hot Start, and 5 µl of first-step DNA. The thermocycling profile was as follows: 15 minutes at 95°C, followed by 30 cycles of denaturation at 94°C for 20 seconds, annealing at 58°C for 30 seconds, and elongation at 72°C for 30 seconds, ending with a final elongation at 72°C for 5 minutes.

Finally, the PCR products were visualized on a 2% (w/v) agarose gel to check for the expected size of the PCR product (Table 1).

Name	Primer sequence (5'-3')	PCR product
PRRS REV RT	TCG CCC TAAT T	
PRRS DIR COM	ATG GCC AGC CAG TCA ATC	
PRRS REV AM	GGC GCA CAG TAT GAT GCG TAG	282 bp
PRRS REV EU	GAT TGC AAG CAG AGG GAG CGT TC	181 bp
PRRS INT I	CAG CCA GTC AAT CAG CTG TG	153 bp
PRRS INT II	*GGT CTG GGT GAG GTG GTG*	

Table I. Specific primers used in this study.

Results

During the experiment, two pigs died 10 weeks after their introduction into the herd. As a result, complete data were obtained for only 33 animals.

In total, 1,236 samples were screened for the presence of PRRSv by RT-nPCR: 700 samples were collected in the first two months (from 35 animals), 330 samples in the third month (from 33 animals), and 206 process controls were equally distributed. In all positive samples, the viral genomes corresponded to the European PRRSv strain. Table 2 and Figure 1 show the data obtained, in numbers and percentages, respectively.

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RT-nPCR positive samples for *PRRSv*	Weeks post introduction/exposure					
	T1	T2	T3	T4	T5	T6
BALF	46	19	151	89	30	20
Oral fluid	19	70	70	70	35	30
Serum	75	60	121	40	10	5
Bronchial swab	124	100	124	151	101	64
Nasal swab	70	54	140	60	74	20
Pigs positive for at least one matrix	33	28	34	35	29	19

Table II. Number of RT-nPCR positive samples (BALF, oral fluid, serum, tracheobronchial swab, and nasal swab PRRSv) from pigs sampled at different times after introduction into the fattening pig unit: T1 (35 pigs): 2 weeks after introduction; T2 (35 pigs): 4 weeks after introduction; T3 (35 pigs): 6 weeks after introduction; T4 (35 pigs): 8 weeks after introduction; T5 (33 pigs): 10 weeks after introduction; T6 (33 pigs): 12 weeks after introduction.

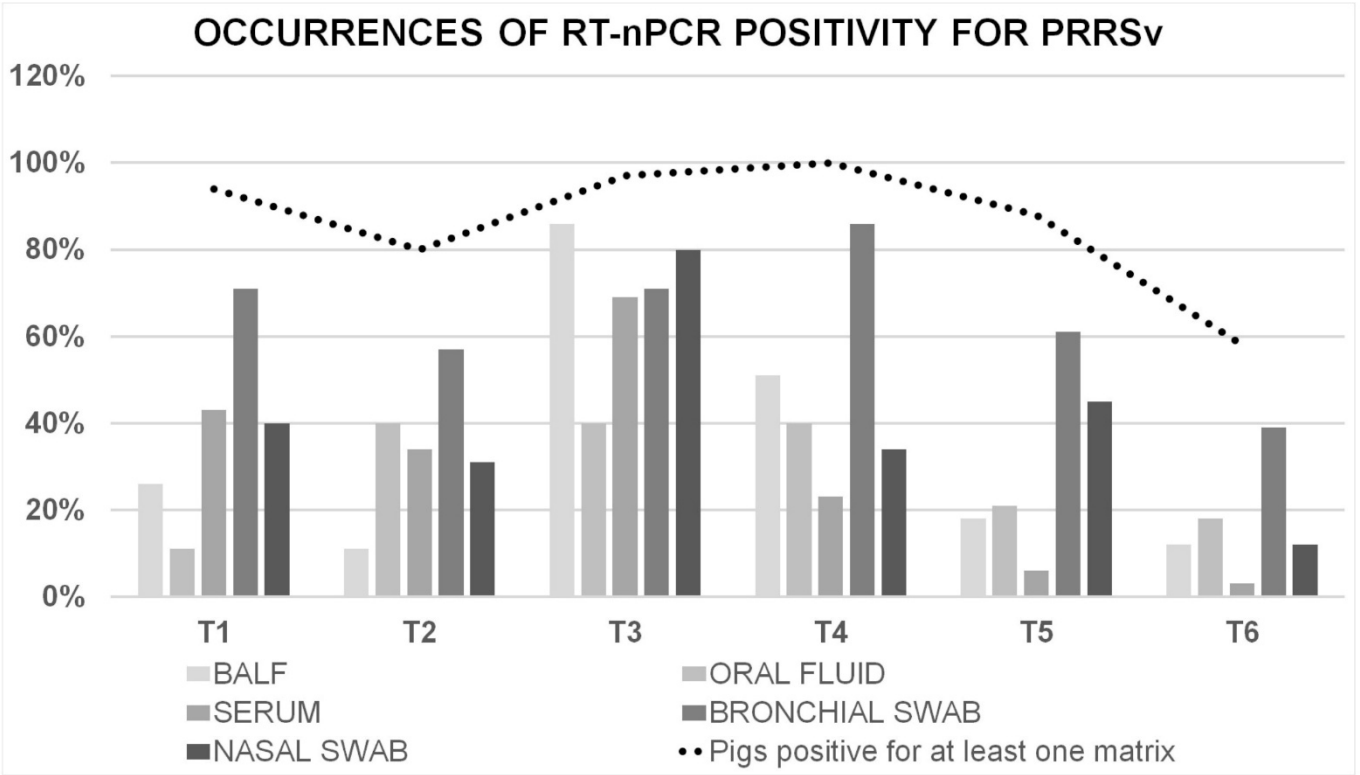

Figure 1. Percentages of RT-nPCR positive samples (BALF, oral fluid, serum, tracheobronchial swab, and nasal swab for PRRSv) and percentages of pigs positive for at least one matrix.

Evaluation of the sensitivity of the biological samples

Table 3 and Figure 2 display the percentages of animals positive for at least one of the five matrices (apparent prevalence, indicated by the dotted line) and the percentages of animals positive for each matrix (relative sensitivity). Confidence intervals are provided for the frequencies in the prevalence data. BALF and tracheobronchial swab showed the highest positivity for PRRSv by RT-nPCR at two different sampling times (T3 and T4).

Throughout the entire study period, tracheobronchial swabs demonstrated the highest sensitivity for PRRSv detection, while oral fluid showed lower diagnostic performance. To calculate the apparent prevalence, a pig was considered

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positive if at least one sample tested positive by RT-nPCR. The prevalence of positive pigs varied from 58% to 100% (Table 3). Eight weeks after the introduction into the fattening pig unit (T4), PRRSv was detected by RT-nPCR in at least one matrix in 100% of the pigs.

During the study, viremic pigs were 43% at T1, decreased to 34% at T2, and then increased to 69% at T3. After reaching its peak, the virus prevalence in serum decreased rapidly.

Time of sampling	BALF+ Pigs % (CI95%)	Serum+ Pigs	broncoalveolar swab+ Pigs	BALF and/or broncoalveolar swab + Pigs	Serum and/or BALF + Pigs	Serum and/or broncoalveolar swab + Pigs	BALF and/or nasal swab + Pigs	Total positive Pigs % (CI95%)
T1	26% (12-43%)	43% (26-61%)	71% (54-85%)	80% (63-92%)	54% (37-71%)	86% (70-95%)	57% (39-74%)	94% (81-99%)
T2	11% (3-27%)	34% (19-52%)	57% (39-74%)	66% (48-81%)	40% (24-58%)	69% (51-83%)	34% (19-52%)	80% (63-92%)
T3	86% (70-95%)	69% (51-83%)	71% (54-85%)	**94% (81-99%)**	**94% (81-99%)**	89% (73-97%)	89% (73-97%)	97% (85-100%)
T4	51% (34-69%)	23% (10-40%)	86% (70-95%)	**94% (81-99%)**	69% (51-83%)	86% (70-95%)	69% (51-83%)	100% (90-100%)
T5	18% (7-34%)	6% (1-19%)	61% (42-76%)	70% (54-85%)	21% (8-37%)	61% (42-76%)	55% (37-71%)	88% (73-97%)
T6	12% (3-27%)	3% (0-15%)	39% (24-58%)	42% (26-61%)	15% (5-30%)	39% (24-58%)	24% (10-40%)	58% (39-74%)

Table III. Percentages of animals positive for at least one matrix, with corresponding confidence intervals.

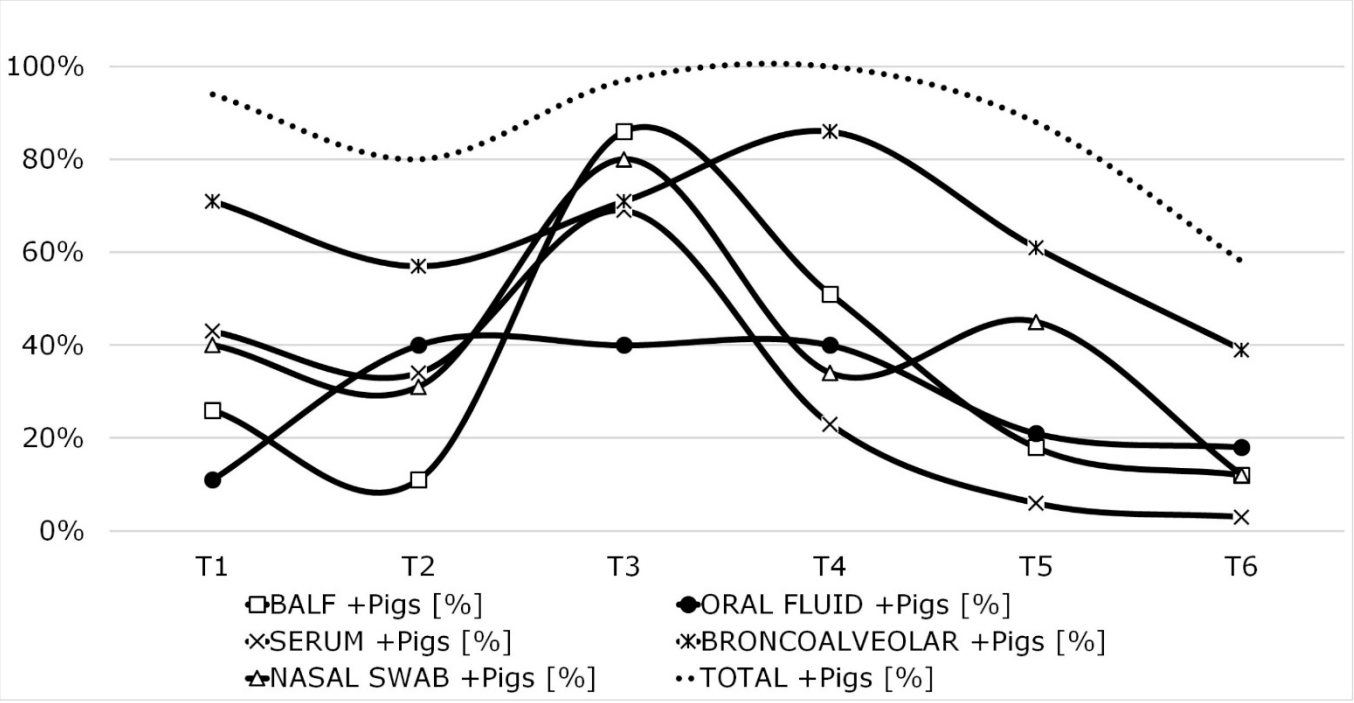

Figure 2. Percentages of animals positive for at least one matrix.

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Relative sensitivity evaluated in parallel in two paired matrices

The parallel evaluation of results obtained from two paired specimens increases the relative sensitivity. Table 3 (gray bars) shows that at T3 and T4, the relative sensitivity increases up to 94% when the results from BALF and bronchial swabs are evaluated together. At T3, the combined analysis of serum and BALF also reaches a relative sensitivity of 94%.

Discussion

The data obtained using PCR technique demonstrated viral circulation in the herd. In the three months following the introduction of animals into the fattening pig unit, all animals included in the study came into contact with PRRSv and developed viremia. Therefore, all tested animals were used as the gold standard for the purposes of this study.

The prevalence rate in blood samples was 43% at T1, decreased to 34% at T2, and then increased to 69% at T3. This pattern suggests the possibility of successive waves of viral circulation among pigs. We hypothesize that newly viremic animals or those in the declining phase of infection were introduced into the herd. These animals acted as reservoirs of infection and were further stimulated by stresses caused by their transition to the new farm. The optimal time for collecting biological samples and obtaining the most accurate estimate of diagnostic performance corresponds to 8 weeks after the introduction of animals into the fattening pig unit (around 18 weeks of age). During this period, all RT-nPCR assays in this study detected at least one PRRS-positive sample for each animal.

The differences in sensitivity between biological matrices likely depend on multiple factors, including: the stage of infection, the donor site, individual variation, the presence of limiting factors in the matrix (e.g., enzymatic degradation of RNA in oral fluid), varying extraction methods using different amounts of the matrix, and the intrinsic extraction efficiency of the kits. Three out of five matrices—namely serum, nasal swabs, and BALF—show a positive peak at T3, highlighting the acute infection phase.

BALF showed the best diagnostic performance by RT-nPCR at T3. In healthy pigs, the cellular composition in BALF is predominantly made up of pulmonary alveolar macrophages (PAMs), with low rates of lymphocytes, and polymorphonuclear neutrophils, eosinophils, and basophilic granulocytes in Specific Pathogen-Free (SPF) animals. Cells from the bronchial epithelium are rare (<1%) and are therefore disregarded in differential counts (Ganter et al., 1997). During PRRS infection, the percentage of PAMs drops after 14 days post-infection (dpi) and then increases at 35 dpi. Conversely, the percentage of lymphocytes in BALF increases from 21 dpi to 35 dpi (Shibata et al., 1997).

As reported by Shibata et al., the intracellular antigens of PRRSv are detected both in PAMs and in SF (BALF supernatant) mainly between 7 and 49 dpi, while they are detected in serum only between 7 and 21 dpi. PRRSv persists longer in PAMs and SF (supernatant sample of BAL) compared to serum after inoculation (Shibata et al., 1997). This suggests that the virus in alveoli is less affected by circulating antibodies compared to the virus in serum.

Although BAL is a very localized sampling technique, potentially not representative of the entire lung, Reinholed et al. (2005) and Moorkamp et al. (2008) state that RT-nPCR analysis of BALF from a caudal lung lobe is comparable to analyzing the entire lung tissue, with the great advantage that it can be performed in vivo on the animal (Scollo et al., 2011). This could explain the high capacity of BALF to detect the virus in the respiratory tract during the early stages, and even up to a few weeks after the end of the viremic phase (Mengeling et al., 1995). This is why a recent study in humans (Gidaris et al., 2010) suggests the use of BAL for the early diagnosis of lung inflammation.

Nasal swabs present a peak of positivity at T3. Previous studies confirm that the amount of virus excreted in nasal mucus increases very rapidly, reaching its maximum at 2 dpi, and then progressively decreases up to 48 dpi (Ruiz et al., 2009).

During the 6 sampling phases, the matrix with the best diagnostic performance was the tracheobronchial swab, even with low quantities of virus. Compared to nasal swabs, this matrix comes from deeper parts of the respiratory tract. However, unlike BALF, it is less sensitive during the viremic phase because it does not sample alveolar macrophages. The intrinsic composition of the bronchial swab may explain why it was able to reveal PRRSv for a longer period compared to other matrices. The swab contains tracheobronchial secretions from a long stretch of the respiratory tract and may contain oropharyngeal contamination (i.e., traces of epithelial cells, oral fluid, and nasal secretions). We hypothesize that these biological secretions likely carry a high viral load.

Although there are conflicting results, several studies have observed PRRSv-positive bronchial epithelial cells (Done et al., 1995) and nasal mucosa cells (Rossow et al., 1996). According to Rutherford et al. (1993), these cells may

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represent migrant monocytes/macrophages with a similar morphological phenotype. Furthermore, we suggest that tracheobronchial swabs may contain the virus present in the epithelium of the crypts and in the follicles of the tonsils (Halbur et al., 1996).

In our data, oral fluid exhibited the worst diagnostic performance. Despite using a stabilizer for sample storage, the temporal trend of positivity exhibited a plateau phase, while other matrices showed an increase in positivity. This behavior may be due to the difficulty in optimizing the extraction phases of viral RNA from this matrix (Van Hout et al., 2013).

To improve diagnostic performance, the results from two different matrices were evaluated in parallel. By applying this approach, we found that in the first 4 weeks after the pigs' entrance into the fattening unit, the highest sensitivity was achieved by parallel testing of serum and tracheobronchial swabs. This is because the virus was detected in both serum and bronchial swabs in all withdrawals.

We obtained higher sensitivity when evaluating BALF and tracheobronchial swabs in parallel one month after mixing pigs. As mentioned earlier, both tracheobronchial swabs and BALF proved to be optimal matrices for detecting PRRSv, as they come from deeper parts of the respiratory tract compared to nasal swabs (Fablet et al., 2011).

Furthermore, since these matrices remain positive for a longer period than the others, the diagnostic window is wider, resulting in a significant improvement in diagnostic performance.

The interpretation of serum and BALF in parallel achieved a sensitivity comparable to the combination of BALF and tracheobronchial swabs only at T3; therefore, the two matrices are well-suited to detect the virus during the acute phase of a PRRS outbreak. This is justified by ongoing viremia combined with the peak sensitivity of BALF, which is the matrix with the highest concentrations of infected alveolar macrophages.

Conclusions

The results of this study indicate that detecting PRRSv in blood samples alone using the RT-nPCR test is insufficient for determining PRRS virus infection. We compared four alternative sampling techniques for PRRSv detection: nasal swabbing, oral sampling, tracheobronchial swabbing, and BALF. RT-nPCR on tracheobronchial swabs proved to be the most effective diagnostic approach for detecting carriers. These alternative specimens are advantageous, as they are easy to collect and minimally stressful for the animals.

Oral fluid collection appeared to be the least sensitive method. Additional validation studies are needed to assess the diagnostic value of oral fluid samples in practice. Furthermore, we explored testing two different matrices in parallel to improve the chances of detecting infected animals. The combination of BALF and tracheobronchial swabs was the most efficient method for detecting PRRSv. However, the BALF collection technique is labor-intensive and stressful for the animals. A valid alternative could be parallel testing of tracheobronchial swabs and serum. Tracheobronchial swabs proved to be a more sensitive matrix than serum, likely because they detected PRRSv in the respiratory tract even after the viremic phase had ended.

Our study suggests that tracheobronchial swabbing combined with RT-nPCR could be an accurate diagnostic tool for assessing infection dynamics in pig herds.

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Ethical approval

Not applicable.

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Conflict of interest

The Authors declare that they have no conflict of interest.

Author contributions

Conceptualization: S.M., M.F., M.G.; Methodology: M.F., S.M., M.G., N.V., I.M.; Formal analysis: S.M., N.V.; Investigation: M.F., S.M., I.M., F.R.; Writing original draft preparation: R.Z., A.T., M.M.; Writing, review and editing: S.P., A.T., M.M.; Visualization: R.Z., A.T., M.M.; Supervision: S.P., M.G., A.D. All Authors have read and agreed to the published version of the manuscript.

Data availability

All data are available upon reasonable request to the corresponding Author.

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