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Assessment of disease-associated *Streptococcus suis* (DASS) carriage and viral co-infections as risk factors for *S. suis* disease outbreaks on German and Dutch farms (2021–2022)

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Abstract

Background
Disease-associated *Streptococcus suis* (DASS) is linked to invasive disease in pigs, yet how carriage of DASS predisposes animals to disease remains poorly understood. Viral co-infections with porcine reproductive and respiratory syndrome virus (PRRSV), swine influenza virus (SIV), and porcine circovirus type 2 (PCV2) have been suggested as additional risk factors. We investigated whether DASS carriage and/or concurrent viral infections were associated with *S. suis* disease outbreaks in a longitudinal field study on three Dutch and three German nursery farms. Three consecutive batches were followed per farm and sampling was initiated when two or more animals presented with clinical signs consistent with *S. suis* disease (outbreak batch). Clinically affected case animals and apparently healthy pen-mates were sampled alongside healthy pigs from an unrelated pen. In subsequent batches without an outbreak, healthy control animals were sampled. Animal classifications were retrospectively verified by post-mortem examination and *S. suis* culture.

Results
S. suis disease was confirmed in 12 of 18 examined batches, with serotypes 9, 2 and 7 being the most frequently isolated from lesions. All Dutch farms were positive for serotype 9. Interestingly, systemic isolates of different serotypes were found in consecutive outbreaks on the same farm. Among necropsied pigs clinically classified as cases, *S. suis* disease was confirmed in 75% in the Netherlands and only 33% in Germany. DASS was detected on all six farms, with batch-level prevalences ranging from 26 to 95%. DASS carriage was not elevated in cases compared to non-diseased animals, although a significantly higher DASS/total *S. suis* ratio was observed in outbreak batches on two farms. Neither metagenomic sequencing nor qPCR revealed a consistent association between viral presence and *S. suis* outbreak status.

Conclusions
No clear risk factors for *S. suis* disease outbreaks were identified. Discordance between clinical and post-mortem diagnosis, a limited number of cases, and imbalanced outbreak and control batches likely contributed to these null findings. Preceding viral or bacterial infections were not assessed here but should be considered in future study designs. These findings highlight the complexity of *S. suis* disease and the considerable challenges of field-based risk factor studies.

Keywords

Disease-associated *Streptococcus suis* (DASS), porcine reproductive and respiratory syndrome virus (PPRSV), swine influenza virus (SIV), porcine circovirus 2 (PCV2), porcine circovirus 3 (PCV3), metagenomic sequencing

1. Background

Streptococcus suis is an ubiquitous Gram-positive bacterial pathogen of pigs that can cause severe disease outbreaks with substantial economic losses, particularly in nursery pigs and occasionally in suckling piglets (1, 2). The bacterium is a constituent of the commensal microbiota of the upper respiratory tract (including the oropharynx, tonsils, and nasal mucosa), as well as the genital and gastrointestinal tracts (3). Piglets become colonised by *S. suis* early in life, either during farrowing or during the rearing period and colonisation rates approach 100% (4, 5). Disease may arise through a poorly understood transition from a colonising to an invasive state, which may involve bacterial penetration of mucosal barriers — such as the tonsillar epithelium — followed by bacteraemia (6). Disease outcome reflects the interaction of microbial, host, and environmental factors (7), all of which vary between farms and over time, making outbreak prediction challenging, although high-risk

groups may be identifiable (8). Risk factors are numerous, differ between studies, and will be discussed in the following sections.

S. suis is a highly diverse pathogen, and strain characteristics are important determinants of its capacity to cause disease. Comparative genomics of more than 3,000 *S. suis* strains isolated from various anatomical sites of healthy and diseased individuals has enabled the identification of pathogenic lineages and pathogenicity markers (9, 10). A conserved genetic marker (virulence marker 1 (VM1)), located within a pathogenicity-associated genomic island highly enriched in disease-associated *S. suis* lineages, has been developed as a qPCR target to assess carriage of disease-associated *S. suis* (DASS) in pig herds. In two farms in the United States, the percentage of DASS positive animals was highly variable across age groups and farms, reflecting farm-specific colonisation dynamics (5). In that study, DASS prevalence at colonisation sites was not correlated with farm mortality; however, both parameters were assessed during the preweaning period, whereas disease outbreaks typically manifest postweaning. Extending these findings to a European context, a more recent study found that DASS was detected at lower frequency and abundance on farms without *S. suis* disease cases in the preceding six months (10), suggesting that DASS prevalence and abundance may have value as a farm-level risk indicator.

The composition of the tonsillar microbiome may also influence the probability of *S. suis* disease. An abundance of certain bacterial species and reduced microbiota diversity during the preweaning phase, may increase the risk of *S. suis* disease in the postweaning phase (11, 12). In addition to other bacterial species, the tonsils also harbour a high diversity of (non-pathogenic) *S. suis* strains (9, 13). Although the influence of the colonizing *S. suis* community on susceptibility to disease remains unclear, this may represent an interesting research topic for future studies.

Host immune responses play a critical role in susceptibility to *S. suis* disease, and animals are typically most vulnerable during the nursery phase, between 4 and 10 weeks of age (14). The elevated incidence of *S. suis* infections during this period coincides with a transient reduction in humoral immunity, characterised by declining maternally derived antibody levels alongside incompletely developed acquired immune responses (15). Maternal factors may also play a role in this immunological window of susceptibility: sow parity influences microbiota development in piglets (16) and affects DASS carriage rates in their offspring (5). A recent study showed that piglets born to gilts carry a higher risk of disease than those born to multiparous sows(8), possibly reflecting differences in the passive transfer of maternal immunity and differences in microbiota composition compared with older sows.

Field observations have associated viral co-infections as significant risk factors of *S. suis* disease outbreaks (17). Porcine reproductive and respiratory syndrome virus (PRRSV), swine influenza virus (SIV), and porcine circovirus type 2 (PCV2) have each been associated with *S. suis* disease in field settings (8, 18, 19). Proposed mechanisms include impairment of macrophage function by PRRSV (20), disruption of respiratory epithelial integrity by SIV (21), and immunosuppressive effects of PCV2 (19). Experimental co-infection studies in pigs generally support these field observations: co-infection with PRRSV enhanced clinical disease and/or increased mortality in *S. suis*-infected pigs(22-24), co-infection with SIV H1N1 exacerbated pulmonary pathology compared with *S. suis* mono-infection (25), while co-infection with PCV2 showed a more modest adverse effect on subsequent *S. suis* disease outcomes (26). However, several important caveats apply: the PRRSV

studies were conducted with highly pathogenic strains rather than European PRRSV genotype 1, and the SIV and PCV2 findings each rest on a single experimental study. Knowledge regarding co-infections with other viruses - and bacteria - remains limited (17).

We hypothesised that DASS carriage and concurrent viral infections constitute a high-risk scenario for *S. suis* disease outbreaks in nursery pigs. To test this hypothesis, we conducted a case-control study on three German and three Dutch farms with a documented history of *S. suis* disease, in which we investigated DASS carriage in the oropharynx and the prevalence of porcine viral pathogens in outbreak versus non-outbreak batches. In addition, consecutive *S. suis* disease outbreaks were characterised through histopathology, *S. suis* culture and serotyping, and analysis of acute-phase protein responses.

2. Material and methods

Selection of study farms

Farms were included in the study if 1) recurrent *S. suis* disease had been recorded on the farm in the two years prior to the study start, 2) no *S. suis* autovaccines were applied at the timepoint of inclusion and 3) the farmer was motivated to inform the veterinarian of an outbreak and allow sampling before treatment.

Farm characteristics

In total, three German (G1, G2 and G3) and three Dutch (N1, N2 and N3) farrowing farms were included and sampled between May 2021 and February 2022. Farm N1 with 700 sows had a one-week farrowing interval. Farm N2 with 280 sows had a five-week farrowing interval, and farm N3 with 370 sows had a two-week farrowing interval. Sows on all Dutch farms were vaccinated against PRRSV, parvovirus and erysipelas. Influenza vaccination was performed only in farm N1 and N2. Sows on farm N1 and N3 also received vaccination against neonatal enteric diseases (i.e. *Clostridium perfringens* (N1), *Escherichia coli* and bovine rotavirus/coronavirus (N1 and N3)) once at approximately day 100 of gestation. Furthermore, sows in batches 2 and 3 of farm N1 received an autogenous *Streptococcus suis* serotype 2 vaccine. The piglet vaccination scheme in farm N1 included vaccination against PCV2 and *Mycoplasma hyopneumoniae* in all batches and against PRRSV in batches 1 and 3. Piglets in farm N2 were vaccinated against *Mycoplasma hyopneumoniae*, proliferative enteropathy, and PRRSV. In farm N3, piglets were vaccinated at three weeks of age against PRRSV, PCV2, and *Mycoplasma hyopneumoniae*. Replacement gilts and boars in farm N3 were vaccinated against PRRSV only. Management procedures differed between the Dutch farms. In farm N2, piglets were not handled within the first 24 hours after birth. An electrolyte solution was offered, tails were docked, teeth were not ground, and male piglets were not castrated (boar fattening). In farm N3, sows were washed with a foam cleaner before entering the farrowing pens. Piglets were not handled within the first 24 hours after birth, tails were docked, teeth were ground, and male piglets were not castrated (boar fattening).

German farms had three-week (G1 and G2) or two-week (G3) farrowing intervals, with 250, 300, and 220 sows, respectively. Sows on all German farms were vaccinated against PRRSV, parvovirus, and erysipelas. On farms G1 and G2, sows were additionally vaccinated against influenza. Piglets on all German farms were vaccinated against PCV2 and *Mycoplasma hyopneumoniae*. Piglets on farms G2 and G3 additionally received vaccination against oedema disease (Shiga toxin 2e) on day 4 of life. Management procedures also differed between the German farms. On farm G1, suckling piglets were not handled within the first 24 hours after birth. Tail docking, teeth grinding, and surgical castration of male piglets were performed within the first week of life. On farm G2, piglets were offered water and an electrolyte solution immediately after birth, and teeth grinding was not performed. Piglets in farms G2 and G3 were neither tail-docked nor castrated (boar fattening) and

remained in the farrowing pens for an additional three to five days after the sows had been removed. On farm G3, sows were washed with a foam cleaner before entering the farrowing pens, split suckling was performed for approximately 1.5 hours at the end of farrowing, and teeth grinding was carried out only in piglets born to gilts.

Outbreak definitions and animal inclusion

Sampling was initiated at included farms when two or more animals presented with clinical signs of *S. suis* disease within 24 hours, which we defined as a *S. suis* disease outbreak (batch 1). Up to five clinically ill animals (case animals CA) showing either a body temperature above 40°C, central nervous disorders, or lameness were included. In addition, up to five apparently healthy animals from the same pen (case-associated control animals CAC) were sampled. In other pens within the same department, another 10-20 apparently healthy pigs were sampled (case non-associated control animals CNAC). After this first sampling, farms were followed up for two consecutive nursery periods (batch 2 and 3). The age and the department of the pigs sampled in batch 1, predetermined the age and department of sampling for batches 2 and 3. When no additional outbreaks occurred within 1 week of the pre-defined sampling time of batch 2 or 3, a total of 20-30 apparently healthy animals (control animals CON) were sampled. If a disease outbreak occurred within 1 week after the sampling, diseased animals and clinically healthy pen-mates were sampled additionally and the period was designated as an outbreak period. The same sampling protocol was applied across all three nursery batches on all six farms, except that necropsies were not performed in batches without an *S. suis*-related disease outbreak.

On-farm sampling and sample processing

CA and CAC animals were anaesthetised before sampling for welfare reasons, while healthy CNAC and CON animals were sampled without sedation. Blood was collected by venapuncture in tubes without anticoagulant and allowed to clot at room temperature. The left and right tonsillar surfaces of each animal were sampled with individual swabs by applying gentle pressure while rotating the swab (eSwab®, Cat no. 490CE.A, Copan, Brescia, Italy) or while holding the swab against the tonsillar surface for 3 seconds (HydraFlock® Swabs (Cat. #25-3206-H 20mm breakpoint), Puritan, USA). After sampling, the eSwabs were snapped off and stored in tubes containing Amies transport medium. The HydraFlock® swab head was inserted into PowerBead Tubes (Cat. #12888-50-PBT, MoBio, Qiagen) prefilled with 750 µL of Power Bead lysis solution and garnet beads (0.7 mm Ø size). All samples were cooled to 4ºC for transport to the laboratory. When sampling was completed, CA and CAC animals were euthanized by intravenous injection of T61 (Intervet Deutschland GmbH, Unterschleissheim, Germany) and submitted to the diagnostic laboratory for necropsy. Upon arrival at the laboratory, swab fluids were aliquoted and stored at -80°C. Swab fluids designated for viral RNA extraction were stored at -80°C in the presence of 66% v/v Trizol LS (Ambion life technologies, Carlsbad, USA). Swabs designated for bacterial culture were shipped at room temperature (RT) to the diagnostic laboratory and, when applicable, stored at 4ºC until culture. Serum was prepared from blood tubes by centrifugation for 10 minutes at 1000xg and stored at -80°C.

Necropsy and Pathological examination

On-farm euthanized pigs were submitted for necropsies to accredited laboratories. Necropsies were performed on 75 pigs classified clinically as either CA (n=34) or CAC (n=41) according to a pre-defined protocol. All Dutch CA animals were necropsied (n=16) and a selection of the German CA animals (n=3/5 CA animals per batch, n=18 in total) were necropsied. Macroscopic findings were recorded and a standardized panel of swabs (eSwab®, Cat no. 490CE.A, Copan, Brescia, Italy) and tissue samples were collected in accordance with the necropsy protocol for pathogen detection and histopathological examination. After opening the skull, the space between cerebellum and cerebrum was swabbed. All joints were opened and examined. In case that no joint was affected, the right tarsal joint was sampled routinely. The pleural cavity was opened and a pleural fluid swab samples taken, the lung and heart were removed and examined and from the lungs an endobronchial swab

sample and tissue specimens collected from the right cranial and left middle lobe. Additional tissue specimens were obtained from the liver, spleen, tonsil, inguinal lymph node, thoracic lymph node and the right frontal and left caudal cerebrum and the cerebellum. Tissue samples were fixed in 10% neutral buffered formalin for at least 24 hours and were embedded after trimming and dehydration in paraffin wax following routine protocols. Tissue sections (4 µm thickness) were stained with haematoxylin-eosin (H&E) and evaluated for histopathological changes with particular attention to inflammatory lesions, by a veterinary pathologist.

Bacteriological examination

From oropharyngeal swabs and swabs from necropsy sites (eSwab®, Cat no. 490CE.A, Copan, Brescia, Italy) 10µl of the liquid Amies medium was plated on selective blood agar (Thermofisher, Wesel, Germany) supplemented with 7.5 mg/l nalidixic acid and 5.0 mg/l colistin sulphate (Thermofisher, Wesel, Germany) and 7% sheep blood using the quadrant streaking method. Plates were incubated at 37°C. In parallel, 100µl of Amies medium was inoculated into non-selective nutrient broth (sodium chloride 5g/l, peptone 10g/l and meat extract 10g/l, adjusted to pH 7.2-7.4). The broth was incubated at 37°C for 20-22 h and then an aliquot was quadrant streaked on Columbia sheep blood agar (CSB; Thermofisher, Wesel, Germany). Cultures were screened for growth of alpha-haemolytic growth suspicious for *S. suis* after overnight incubation and after 46-48 hrs incubation.

For all oropharyngeal and necropsy samples up to 12 different alpha-haemolytic colonies were passaged on CSB and preliminarily identified by MALDI-MS (Biotyper; Bruker, Bremen Germany; MBT Compas Library RUO Version 10). Per sample, up to three individual cultures identified as *S. suis* with score values ≥2 were stored in brain heart infusion broth (Becton Dickinson, Heidelberg, Germany), supplemented with 25% glycerol (Merck, Darmstadt, Germany), 50% heat inactivated horse serum (TSC Biosciences, Buckingham, UK) and 3% glucose (Merck, Darmstadt, Germany) at -80°C. For each farm at least one representative isolate from a systemic compartment of a diseased pig was serotyped by PCR in advance to whole-genome sequencing (27).

Final classification of animals

The clinical classification of animals as CA, CAC, CNAC and CON was retrospectively verified by pathological and bacteriological findings. The post-mortem diagnosis was defined as the gold standard. A true CA (trCA) was characterized by 1) positive bacteriological culture of *S. suis* from normally sterile body compartments (brain, cavities or joints) and 2) macroscopic and/or microscopic inflammatory signs consistent with *S. suis* disease. If a pig categorized as CAC (based on a lack of clinical symptoms) had positive *S. suis* culture from respective organs accompanied by pathological changes described above, it was re-assigned trCA. Animals from the same batch were re-assigned trCAC, trCNAC and trCON according to the location of trCA animals. Pigs were re-assigned to the final category “other“(OTHER) when they were designated as CA or CAC by clinical signs and location in the farm, but *S. suis* disease could not be confirmed by post-mortem diagnostics.

Whole genome sequencing and in silico serotyping

S. suis isolates cultured from systemic compartments (brain, joint, cavities) of trCA animals were subjected to Illumina whole genome sequencing. DNA extraction, library preparation, and sequencing were undertaken using a HiSeq 2500 instrument (Illumina, San Diego, CA, USA) by MicrobesNG (Birmingham, UK). Raw sequence reads were pre-processed using Trimmomatic V0.30 to remove adaptors, trim poor-quality ends, and delete short sequences (<36 nt) (28). Raw and pre-processed reads were assessed for quality to ensure cleaning efficiency using FastQC (Babraham Bioinformatics). Isolates with low quality reads were excluded from the collection. We generated de novo assemblies with Spades v.3.57 (29). The isolates were grouped into clusters of closely related genomes using PopPUNK (v2.6.5). The majority of isolates were grouped in clonal clusters of a shared PopPUNK lineage, and sequencing reads were mapped to a reference strain assembly from

the same or a closely related cluster using Bowtie2 (v2.2.6). More divergent isolates belonging to diverse PopPUNK clusters were retained as de novo assemblies. Serotypes were determined in silico from assemblies using the Athey et al. pipeline (30).

qPCR detection and quantification of total *S. suis* and DASS

Total *S. suis* and disease-associated *S. suis* (DASS) were quantified using the 16S rRNA and vm1 qPCR targets, respectively. Vm1 corresponds to the disease-associated marker 1 (DAM1) (SSU_RS01130) described by Gussak et al. (10).” DNA isolated was from oropharyngeal swab samples (HydraFlock® Swabs (Cat. #25-3206-H 20mm breakpoint, Puritan, USA)). The DNA extraction and qPCR conditions were as described by Gussak et al. (10). A positivity cut-off of 40 copies/reaction was used for 16S rRNA qPCR and 10 copies/reaction for vm1-qPCR. In all subsequent analysis 16S rRNA qPCR copy numbers were corrected for the abundance of 16S rRNA target (4 copies per *S. suis* genome). In addition to analysing vm1-copy numbers to assess DASS-quantity, the DASS/*S. suis* copy number ratio was calculated to account for variation in the amount of material caught on oropharyngeal swabs.

Viral detection by Targeted Capture of Viral Sequences

Nucleic acid extraction and generation of ds cDNA

Presence of viral genetic material was assessed in oropharyngeal swab fluid samples (eSwab®, Cat no. 490CE.A, Copan, Brescia, Italy) from 217 animals from 16 of 18 nursery periods (no samples from G3-2 and G3-3). Samples from the same batch were pooled into pools containing 2 to 5 samples. DNA purification was performed using the Qiagen QIAamp MinElute Virus Spin Kit (Qiagen, Venlo,the Netherlands) according to the manual description. Viral RNA was extracted using the Quick-DNA/RNA Pathogen MagBead Kit (Zymo Research) on a PurePrep 96 automated extractor (Molgen), according to the manufacturer’s magnetic-bead protocol. Where applicable, on-bead DNase I treatment was included. Lysates were processed by automated magnetic-bead binding, sequential wash steps, and elution in 30–50 µL nuclease-free water. Sequence-independent single-primer amplification (SISPA) was performed on viral RNA to generate double-stranded (ds) cDNA. First-strand cDNA synthesis used the pan viral A primer and SuperScript III reverse transcriptase (Thermo Fisher Scientific), followed by second-strand synthesis with Sequenase DNA polymerase (Thermo Fisher Scientific). The ds cDNA was subsequently amplified with the pan viral B primer. Products were purified with AMPure XP magnetic beads (Beckman Coulter, Agencourt) and quantified (fluorometric). Exactly 100 ng of SISPA-derived ds cDNA per sample served as input for NGS library preparation.

Library preparation and metagenomic sequencing

Sequencing libraries were prepared from ds DNA using the Roche library preparation kit supplied for ViroCap workflows (Roche) (31), following the manufacturer’s protocol, including end-repair, A-tailing, and adapter ligation. Libraries were quantified using the Quant-iT dsDNA High-Sensitivity (HS) Assay Kit (Thermo Fisher Scientific). Libraries were normalized at 125 ng per sample and pooled to a total of 1,000 ng prior to probe-capture hybridization targeted viral enrichment (ViroCap). Targeted capture of viral sequences was performed using the ViroCap procedure, following the manufacturer’s guidelines without deviation unless specified. In brief, libraries prepared from ds cDNA underwent hybridization with biotinylated capture probes targeting a broad virome panel, followed by recovery with streptavidin-coated beads and post-capture amplification per kit recommendations. Post-capture amplification was performed using kit-supplied reagents, followed by purification with AMPure XP beads (Beckman Coulter, Agencourt). Quality control comprised fragment-size profiling on the TapeStation 2200 (Agilent) and concentration measurement using the Quant-iT dsDNA HS Assay Kit. Pooled libraries were sequenced on the Illumina NovaSeq 6000

(Illumina) to generate paired-end 2×150 bp reads using the Reagent Kit (PE150), targeting approximately 2.0 × 10^6 paired-end reads per sample.

Bioinformatics and taxonomic assignment

Raw FASTQ files were adapter- and quality-trimmed using the BBTools suite (e.g., BBDuk) in combination with in-house developed scripts (32). Polished reads were aligned with BBMap against (i) the NCBI viral reference database (RefSeq Viral) and (ii) the Roche VirCapSeq target reference. Alignments of reads were summarized and exported as per sample tables. In these tables the nucleotide sequence of reads, their unique read count, length and breadth of coverage were provided together with the accession number of the viral ORF/protein sequence (taxonomic assignment) that scored the lowest *E*-value (highest similarity) in BBMap. Candidate detections were manually reviewed in Integrative Genomics Viewer (IGV). Per-sample tables were manually reviewed in order to remove reads i) showing the highest similarity to bacteriophage genomes, ii) reads containing poly C, G, A, T or poly-A-T stretches, and iii) reads containing stretches of concatenated repeat sequences. In addition, all reads that aligned to genomes of viruses not commonly present in porcine species (33, 34) were compared to sequences in the NCBI non-redundant nucleotide and/or protein databases using blastn and/or blastx to evaluate the correctness of the taxonomic assignment generated by BBMap. Reads that showed the highest similarity (lowest *E*-value) to a porcine (host) sequence or bacteriophage sequence in this blastn/blastx analysis were also removed from per-sample tables. If applicable, taxonomic assignments generated by BBMap were replaced by assignments scoring a higher similarity in this blastn/blastx analysis. All remaining reads in per-sample tables were marked as “valid”, and tagged with the taxonomic abbreviation of the viral genome to which they aligned. The presence of a viral genome in pools of oropharyngeal swabs was considered positive if at least 2 “valid” reads aligned to different ORF’s within a viral genome, or 2 “valid” reads aligned to different parts of an ORF.

qPCR diagnostics for PRRSV and PCV

Viral RNA and DNA were isolated from oropharyngeal swab fluid (Amies medium liquid stored at -70 in presence of 66% Trizol LS) or serum using DNA and RNA isolation kits (Zymo Research) or the RNeasy Blood and Tissue Kit, (Qiagen GmbH, Hilden, Germany) according to the manufacturer’s instruction. Viral nucleic acids were stored at −70°C until use. The presence of PRRSV-1 (EU) was analysed in serum of 236 pigs representing all 18 study batches using the virotype PRRSV RT-PCR Kit (Indical biosciences, Germany) according to the instructions of the manufacturer. Presence of PCV2 and PCV3 was determined in a selected subset of 77 pigs from 13 batches, comprising of 25 animals from five PCV-2 positive pools and 52 animals from 13 PCV-2 negative pools as identified by metagenomic Virocap analysis. PCR was performed according to methods described elsewhere (35), with the exception that PCV-2 and PCV-3 PCRs were executed as singleplex reactions. Amplifications were performed in a real-time cycler (Applied Biosystems 7500 Real Time PCR System, Thermo Fisher Scientific, Waltham, Massachusetts, USA).

Measurement of acute phase proteins

Acute phase proteins were analysed in serum samples of pigs from various classes (trCA n =20, trCAC n = 18, CNAC n =1, CON n = 4, others n =29) from 13 of 18 nursery periods. The analyses were performed by using immunoperoxidase assays supplied by Life Diagnostics Inc. (West Chester, USA) targeting C-reactive protein (cat. Pig CRP-9) or haptoglobin (cat. Pig HAPT-9) according to the manufacturer’s instructions and using the supplied standards.

Data analysis and Statistical evaluation

Statistical analysis was performed using Graph Pad Prism 10 software. Quantities of CRP, Haptoglobin, *S. suis* and DASS were compared between animals with different classifications using a non-parametric Kruskal-Wallis test, as appropriate by normality testing.

Proportions of DASS-positive and DASS-negative animals within different animal classes were compared using a chi-square test. Outbreak and control batches were compared for presence of viral sequences using Fisher's exact test and Holm-Šídák correction for multiple comparisons when applicable. Within outbreak batches, presence of viral sequences in clinically affected animals (trCA) versus healthy penmates (trCAC) was compared using Fisher's exact test with a Holm-Šídák post-hoc test.

AI-assisted language editing

Claude (Anthropic) was used for language editing and formatting. All content was reviewed and verified by the authors, who take full responsibility for the final text.

3. Results

Characterization of *S. suis*-associated disease outbreaks

Three Dutch and three German farms were followed in 2021-2022 for the occurrence of *S. suis* disease outbreaks and each farm was sampled three times, either during an outbreak or in the absence of an outbreak. Across the Dutch farms, seven batches presented clinical signs of *S. suis* disease during the study period and 191 pigs were sampled (age 7–9 weeks). A total of 16 animals were clinically classified as cases (CA) and necropsied to confirm diagnosis. Across the German farms, *S. suis* disease outbreaks were clinically diagnosed in six batches and 270 pigs were sampled (age 4–5 weeks). A total of 30 were clinically classified as CA, of which 18 were necropsied to confirm diagnosis (max. 3 CA per batch). Furthermore, across both countries, 41 clinically healthy pen-mates of CA animals were necropsied as case-associated controls (CAC). Post-mortem isolation of *S. suis* from brain, joints, brain or body cavities, in combination with macroscopic and/or histological inflammatory findings consistent with *S. suis* disease, was considered the gold standard for identifying true *S. suis* disease cases (see Methods). An overview of animal classification according to clinical signs and final post-mortem diagnosis is presented in **Table 1**. *S. suis*-specific necropsy findings with bacteriological isolation data from different organs are summarised in **Table 2**.

Of the 16 CA pigs necropsied in the Netherlands, 12 were confirmed as trCA (positive predictive value, PPV = 75%). Of the 18 CA pigs necropsied in Germany, only 6 were confirmed as trCA (PPV = 33,3%). Five of the 41 clinically healthy animals (CAC) subjected to post-mortem examination (3 in the Netherlands, 2 in Germany), met the criteria for trCA based on *S. suis* culture in combination with macroscopic and/or histological findings, despite the absence of clinical signs of *S. suis* disease recorded by the veterinarian. In total, 15 trCA were identified in the Netherlands compared to only 8 in Germany. The specific bacteriological and pathological findings in the 23 trCA are listed in **Supplementary Table 1**. All seven batches classified as outbreak batches based on clinical signs in the Netherlands were confirmed as containing at least one trCA by post-mortem diagnosis. In Germany, five of the six batches initially classified as outbreak batches contained a confirmed trCA. One batch without a confirmed trCA (G3-2) was consequently reclassified as control batch.

For further individual-level analyses, CA animals not confirmed as trCA were assigned to a new category designated "OTHER", together with their pen-mates originally classified as CAC. To compare systemic inflammatory responses across groups, concentrations of the acute phase proteins C-reactive protein (CRP) and haptoglobin (Hp) were measured in a subset of animals. CRP and Hp concentrations were significantly higher in trCA pigs than in their clinically healthy trCAC pen-mates ($p < 0.0001$) (**Figure 1**), consistent with a more pronounced systemic inflammatory response. The OTHER group exhibited elevated CRP concentrations relative to the trCA group ($p < 0.05$), indicating that animals in pens clinically marked as diseased by the veterinarian also displayed systemic inflammation.

Figure 1

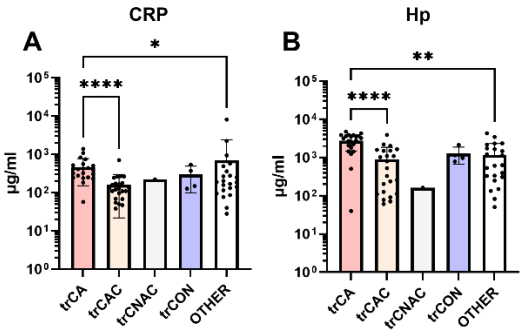


Figure 1: Acute phase protein concentrations in pigs from according to animal classification. A) concentration of C-reactive protein (CRP) and B) Haptoglobin (Hp) in serum of pigs from a subset of trCA (n=22), trCAC (n=23, trCNAC (n=1), trCON (n=4) and OTHER (n=22) animals as determined by ELISA. Concentrations in trCA, trCAC and OTHER groups were compared using a non-parametric Kruskal-Wallis test with Dunn's multiple comparison test. Bars indicate mean values + SD and dots show results of individual animals. Significant differences are indicated by asterisks, with * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001. trCNAC and trCON were not compared because of limited number of animals tested in those groups.

Table. 1: Classification of pigs based on clinical and post-mortem (final-true) examination

	Farm[1]	N1			N2			N3			G1			G2			G3			Total
	Batch	1	2	3	1	**2[a]**	3	1	**2[a]**	3	1	2	**3[a]**	1	2	**3[a]**	1	**2[a]**	**3[a]**	
	Pigs (n)	14	27	24	16	**20**	28	14	**20**	28	30	30	**30**	30	30	**30**	30	**30**	**30**	461
Clinical classification[2]	CA	2	4	3	1	**0**	3	1	**0**	3	5	5	**0**	5	5	**0**	5	**5**	**0**	47
	CAC	2	3	11	5	**0**	5	3	**0**	5	5	5	**0**	5	5	**0**	5	**5**	**0**	64
	CNAC	10	20	10	10	**0**	20	10	**0**	20	20	20	**0**	20	20	**0**	20	**20**	**0**	220
	CON	0	0	0	0	**20**	0	0	**20**	0	0	0	**30**	0	0	**30**	0	**0**	**30**	130
Final (True) classification[3]	trCA	2	1	1	3	**0**	3	2	**0**	3	2	2	**0**	2	1	**0**	2	**0**	**0**	23
	trCAC	2	5	7	3	**0**	6	2	**0**	9	4	5	**0**	7	6	**0**	7	**0**	**0**	62
	trCNAC	10	18	12	10	**0**	16	10	**0**	16	22	19	**0**	19	19	**0**	19	**0**	**0**	181
	trCON	0	0	0	0	**20**	0	0	**20**	0	0	0	**0**	0	0	**30**	0	**24**	**30**	164
	OTHER	0	3	4	0	**0**	3	0	**0**	0	2	4	**0**	2	4	**0**	2	**6**	**0**	31
	Age (w)	8	7	8	7	**7**	7	8	**9**	7	**5**	5	**5**	**5**	5	**5**	4	**4**	**4**	

[1]N and G denote country of origin (N = the Netherlands, G = Germany); the first digit indicates the farm ID [2]The clinical classification of animals as CA, CAC, CNAC and CON as determined by the visiting veterinarian. [3]The final classification of animals after pathological and bacteriological examination post-mortem. A true CA (trCA) was characterized by specific macroscopic and/or microscopic inflammatory signs and positive bacteriological culture of *S. suis* from brain, cavities or joints. Animals from the same batch were re-assigned trCAC, trCNAC

and trCON according to the location of trCA animals. Pigs were re-assigned as OTHER when they were originally designated as CA or CAC, but *S. suis* disease could not be confirmed by post-mortem diagnostics. [a]Columns in bold indicate non-diseased control batches (based on final classification).

Bacteriological findings

S. suis serotypes isolated from normally sterile body compartments of trCA animals are listed in **Table 2** and **Supplementary Table 1.** Serotypes 9, 2, and 7 were most frequently found in outbreaks. Consecutive outbreaks on the same farm involved different serotypes. There was just one outbreak (G3-1) where one exclusive serotype was found in all animals and organs (serotype 2). In other outbreaks involving more than one animal, systemic isolates of different serotypes were detected. In 4/23 trCA animals, different serotypes were also found between or within isolation sites in the same animal. In The Netherlands, serotype 9 was isolated on all farms and was the dominant serotype in outbreaks N1-1, N1-2, N1-3 and N3-3. In Germany there was more variation; different serotypes were found in each outbreak and serotype 9 was found only in one animal (**Supplementary Table 1**).

S. suis serotypes isolated from normally sterile body sites of trCA animals are listed in **Table 2** and **Supplementary Table 1**. Serotypes 9, 2 and 7 were most frequently isolated across all outbreaks. In most outbreaks, more than one serotype was isolated, and in some animals, multiple serotypes were isolated from the same animal. Only one outbreak (G3-1) involved a single serotype (serotype 2) across all animals and organs. In the Netherlands, serotype 9 was isolated on all farms and was the dominant serotype in outbreaks N1-1, N1-2, N1-3 and N3-3. Serotype distribution was more heterogeneous in Germany: the serotype composition differed between outbreaks, and serotype 9 was isolated from only one animal (**Supplementary Table 1**).

Oropharyngeal carriage of total *S. suis* (16S rRNA) and DASS (vm1-positive) *S. suis* was monitored in piglets by qPCR and bacteriological culture. qPCR results are displayed in **Figure 2**. Notably, 98% (323/331) of all oropharyngeal swabs analysed by 16S rRNA qPCR were positive for *S. suis* contrasting with only 60% (224/371) of oropharyngeal swabs as analysed by bacteriological culture. DASS was detected in an average of 63.1% of all tested animals, and prevalences did not differ significantly between animal classes (Chi-square test; **Figure 2A**). DASS was detected on all six farms, with batch-level prevalences ranging from 26 to 95% (**Figure 2B**); however, no significant differences were observed when comparing outbreak and control batch prevalences (Mann–Whitney test, $p > 0.99$).

Bacterial load, expressed as copy number/PCR reaction, was also analysed within DASS-positive and total *S. suis*-positive animals. Adjusted copy numbers ranged from 15 to 129.191 for total *S. suis* and from 10 to 1840 for DASS, with DASS copy numbers averaging 101-fold lower than total *S. suis* copy numbers. Comparison of total *S. suis* copy numbers within farms revealed that certain batches carried higher bacterial loads (**Figure 2C**). DASS quantities did not differ significantly between batches on the same farm (**Figure 2D**). When the DASS/total *S. suis* ratio was calculated, one outbreak batch in farm G2 and in N2 displayed a significantly higher ratio compared to their control batch (**Figure 2E**). This difference however, was not observed in farms G1 and N3. Finally, oropharyngeal *S. suis* and DASS copy numbers were analysed according to the animal classification of the host (trCA, trCAC, trCNAC, trCON, OTHER); no significant differences were observed (**Figure S1**).

Figure 2

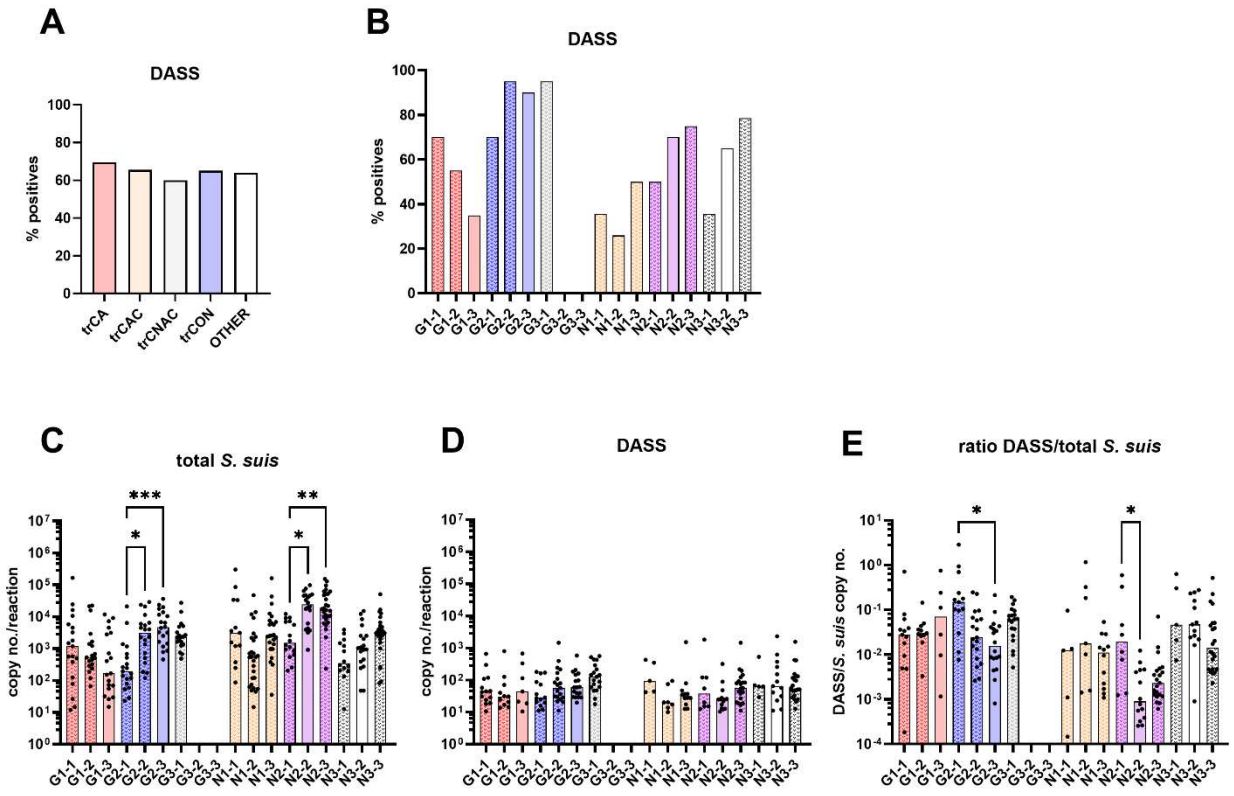


Figure 2: Carriage of DASS and total *S. suis* in the oropharynx.
A) Percentage of DASS-positive piglets in oropharyngeal swabs in trCA (n=23) trCAC (n=58), trCNAC (n=145), trCON (n=80) and OTHER (n=25) groups as determined by vm1-qPCR. B) proportion of DASS-positive animals per batch on Dutch and German farms. Batches G3-2 and G3-3 were not analysed. C) Genome copy numbers of *S. suis* in *S. suis*-positive oropharyngeal swabs from Dutch and German farms as determined by 16S rRNA qPCR. Copy numbers are corrected for the abundance of 16S rRNA target (4 copies per *S. suis* genome). D) Genome copy numbers of DASS in DASS-positive oropharyngeal swabs from Dutch and German farms as determined by vm1 qPCR. E) ratio of DASS copy numbers/*S. suis* copy numbers. Genome copy numbers and ratios were compared between different batches on each farm using a non-parametric Kruskal-Wallis test with Dunn's multiple comparison test. Batches G3-2 and G3-3 were not included in the analysis. Bars depict the median per farm period and individual values are shown with dots. Outbreak batches are indicated by dotted bars. Significant differences are indicated by asterisks, with * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

Table 2. *S. suis* specific pathological and bacteriological findings in necropsied animals.

Batch[1]	N1-1	N1-2	N1-3	N2-1	**N2-2[a]**	N2-3	N3-1	**N3-2[a]**	N3-3	G1-1	G1-2	**G1-3[a]**	G2-1	G2-2	**G2-3[a]**	G3-1	**G3-2[a]**	**G3-3[a]**	Total
Necrop. CA	2	4	2	1	**0**	3	1	**0**	3	3	3	**0**	3	3	**0**	3	**3**	**0**	34
Necrop.total	4	7	8	4	**0**	6	4	**0**	6	6	6	**0**	6	6	**0**	6	**6**	**0**	75
Macroscopic findings[2]																			
Meningitis	2/4	2/7	1/8	1/4	**0**	2/6	1/4	**0**	2/6	0/6	1/6	**0**	0/6	1/6	**0**	0/6	**1/6**	**0**	14/75
Arthritis	0/4	1/7	2/8	1/4	**0**	2/6	0/4	**0**	3/6	0/6	2/6	**0**	1/6	0/6	**0**	2/6	**1/6**	**0**	15/75
Pericarditis	0/4	0/7	0/8	2/4	**0**	0/6	0/4	**0**	0/6	0/6	0/6	**0**	0/6	1/6	**0**	0/6	**0/6**	**0**	3/75
Peritonitis	2/4	2/7	2/8	2/4	**0**	3/6	1/4	**0**	3/6	0/6	0/6	**0**	2/6	1/6	**0**	0/6	**2/6**	**0**	20/75
Pleuritis	2/4	1/7	0/8	2/4	**0**	0/6	0/4	**0**	0/6	3/6	0/6	**0**	2/6	1/6	**0**	0/6	**0/6**	**0**	11/75
Histology findings[2]																			
CNS	2/4	2/6	1/7	2/4	**0**	4/5	1/4	**0**	3/6	0/6	1/5	**0**	0/3	3/6	**0**	1/5	**0/6**	**0**	20/67
Synovialis	n.d.	1/6	0/3	2/3	**0**	1/6	0/4	**0**	0/1	n.d.	1/4	**0**	0/4	0/3	**0**	1/5	**n.d.**	**0**	6/39
Spleen	0/3	0/6	1/7	2/4	**0**	1/6	0/3	**0**	0/6	1/5	1/6	**0**	3/5	1/6	**0**	0/6	**0/5**	**0**	10/68
***S. suis* isolation[2]**																			
Brain	2/4	3/7	2/8	3/4	**0**	2/6	1/4	**0**	3/6	1/6	2/6	**0**	1/6	0/6	**0**	2/6	**0/6**	**0**	22/75
Cavities	0/4	0/7	1/8	2/4	**0**	1/6	2/4	**0**	4/6	1/6	1/6	**0**	1/6	1/6	**0**	1/6	**0/6**	**0**	15/75
Joint	0/4	1/7	0/8	1/4	**0**	0/6	0/4	**0**	3/6	1/6	1/6	**0**	0/6	0/6	**0**	2/6	**0/6**	**0**	9/75
Oropharynx (tonsil)	4/4	7/7	7/8	3/4	**0**	3/6	4/4	**0**	3/6	6/6	0/6	**0**	2/6	4/6	**0**	3/6	**3/6**	**0**	49/75
Serotypes isolated[3]	9, 18	9	9, 16	2, 7	**-**	2, 9	3, 4	**-**	2, 9, 7, 15	15, 29	9, 14	**-**	7	n.t.[4]	**-**	2	**-**	**-**	

[1]N and G denote country of origin (N= the Netherlands, G=Germany), the first digit indicates the farm and the second indicates the batch. [2]Number of pigs with specific findings out of the total pigs analyzed per batch (for same animals histology samples were not available). [3]Serotypes isolated from either brain, joint or cavities (systemic sites). [4]Serotype could not be assigned: not typable (n.t.). [a]Columns with bold text indicate non-diseased control batches.

Concurrent presence of viral nucleic acids

Oropharyngeal swabs from up to five animals from the same batch were pooled and analysed for the presence of viral RNA/DNA sequences using targeted viral sequence capture (ViroCap) and metagenomic sequencing. Results are summarised in **Table 3**. SIV was not detected in any oropharyngeal sample and is therefore not shown. Detection of PRRSV (3/16) and PCV2 (3/16) was exclusive to German farms. Porcine coronavirus (PRCV/TGEV) (2/16) and porcine respirovirus 1 (PRV1) (2/16) were detected in a subset of batches in both countries. All batches were positive for porcine endogenous retrovirus (PERV) and parvo-boca virus. Torque teno sus virus (TTSuV) (6/16) and porcine haemagglutinating encephalomyelitis virus (PHEV) (4/16) were found in both countries, while adenovirus (4/16) and porcine stool-associated circular virus (PoSCV) (2/16) were identified exclusively in Germany. Sequences of viruses commonly associated with enteric infection — astrovirus (14/16), kobuvirus (4/16), sapelovirus (4/16), rotavirus (3/16), sapovirus (2/16), enterovirus G (1/16), and torovirus (1/16) — were also commonly detected across the batches. Reads corresponding to the non-porcine viruses bovine viral diarrhoea virus (BVDV) (8/16) and SARS-CoV-2 (4/16) were also identified. The association between viral sequence detection and *S. suis* outbreak status was examined; none of the viruses showed a significantly higher occurrence in outbreak compared to control batches (**Table 3**).

In addition to the pooled ViroCap analysis, a subset of individual animals was tested for the presence of PRRSV, PCV2, and PCV3 by qPCR in oropharyngeal swab and/or serum (**Figure 3**). PCV3 sequences are not included in the targeted sequence capture panel of ViroCap (37), and qPCR was therefore used to assess the presence of this virus. All batches in which PRRSV and PCV2 sequences were detected by ViroCap also contained qPCR-positive animals viruses (**Figure 3**). On the other hand, qPCR detected positive animals in several batches that were negative by ViroCap, including batches N1-1, N1-2, N3-1, and N3-2 for PRRSV, and batches G2-1, G2-2, G3-1, and N3-1 for PCV2. When comparing qPCR results between batches, PRRSV-positive animals were found in 6/12 outbreak batches versus 3/6 control batches, a difference that was not significant (Fisher's exact test, $p > 0,99$). For PCV2, 6/12 tested outbreak batches contained positive animals, as did the tested control batch. PCV3-positive animals were found in 8/12 tested outbreak batches and not in the tested control batch. Notably, PCV2 was detected more frequently on German farms than on Dutch farms, while PCV3 was detected more frequently on Dutch farms. PCV2 and PCV3 were not detected simultaneously in the same animal, with the exception of one animal from batch G2-2.

When considering only viraemia based on detection of viral nucleic acids in serum (**Figure 3C, D**), PRRSV viraemia was found in 45/236 (19.1%) animals, PCV2 viraemia in 17/77 (22.1%), and PCV3 viraemia in 15/77 (19.5%). Within outbreak batches, animals affected by *S. suis* disease (trCA) were also assessed for whether they had higher PRRSV, PCV2, or PCV3 qPCR positivity rates compared with their healthy pen-mates (trCAC); no differences were found (data not shown).

Table 3. Presence of viral sequences per batch as determined by metagenomic sequencing.

Batch[1]	N1-1	N1-2	N1-3	N2-1	**N2-2[a]**	N2-3	N3-1	**N3-2[a]**	N3-3	G1-1	G1-2	**G1-3[a]**	G2-1	G2-2	**G2-3[a]**	G3-1	Outbreak total	**Control total[a]**	Outbreak vs. control
Pigs (n)[2]	12	13	15	15	**5**	18	11	**5**	15	20	16	**5**	20	20	**5**	20	195	**20**	
Pools (n)	3	3	3	3	**1**	4	3	**1**	4	4	3	**1**	4	4	**1**	4	42	**4**	
Virus (0/1)[3]																	*%pos[3]*	*%pos[3]*	*p-value[4]*
PRRSV	0	0	0	0	**0**	0	0	**0**	0	1	0	**0**	1	1	**0**	0	25	**0**	>0.99
PCV2	0	0	0	0	**0**	0	0	**0**	0	1	1	**1**	0	0	**0**	0	17	**25**	>0.99
PRCV/TGEV	1	0	0	0	**0**	0	0	**0**	0	0	0	**0**	0	0	**0**	1	17	**0**	>0.99
PRV1	0	0	0	0	**0**	1	0	**0**	0	0	0	**0**	1	0	**0**	0	17	**0**	>0.99
PERV	1	1	1	1	**1**	1	1	**1**	1	1	1	**1**	1	1	**1**	1	100	**100**	>0.99
Parvo-boca virus	1	1	1	1	**1**	1	1	**1**	1	1	1	**1**	1	1	**1**	1	100	**100**	>0.99
TTSuV	1	0	0	0	**0**	1	0	**0**	1	1	0	**1**	0	1	**0**	0	42	**25**	>0.99
PHEV	0	1	0	0	**0**	0	1	**0**	0	0	1	**0**	0	1	**0**	0	33	**0**	>0.99
Adenovirus	0	0	0	0	**0**	0	0	**0**	0	1	1	**0**	0	0	**1**	1	25	**25**	>0.99
PoSCV	0	0	0	0	**0**	0	0	**0**	0	0	1	**0**	0	0	**0**	1	17	**0**	>0.99
Astrovirus	1	1	1	1	**0**	1	1	**0**	1	1	1	**1**	1	1	**1**	1	100	**50**	0.62
Kobuvirus	0	1	0	0	**0**	0	0	**0**	0	1	1	**0**	0	0	**0**	1	33	**0**	>0.99
Sapelovirus	1	0	0	0	**0**	1	0	**0**	0	1	0	**0**	0	1	**0**	0	33	**0**	>0.99
Rotavirus	0	0	0	0	**0**	0	1	**0**	0	0	0	**0**	0	0	**1**	1	17	**25**	>0.99
Sapovirus	0	1	0	0	**0**	0	0	**0**	0	0	0	**1**	0	0	**0**	0	8	**25**	>0.99
Enterovirus G	0	1	0	0	**0**	0	0	**0**	0	0	0	**0**	0	0	**0**	0	8	**0**	>0.99
Torovirus	0	0	0	0	**0**	0	0	**0**	0	0	0	**1**	0	0	**0**	0	0	**25**	>0.99
BVDV	0	0	1	0	**0**	1	0	**1**	1	1	0	**1**	0	0	**1**	1	42	**75**	>0.99
SARS-Cov-2	0	1	0	0	**0**	0	0	**1**	0	0	1	**0**	0	1	**0**	0	25	**25**	>0.99

[1]N and G denote country of origin (N = the Netherlands, G = Germany); the first digit indicates the farm and the second indicates the batch. [2] Presence of viral nucleic acids was assessed in oropharyngeal swab fluid samples of 215 animals from 16 nursery periods (no samples from G3-2 and G3-3), samples from the same batch were pooled in pools of 2 to 5 samples. [3]Values indicate the occurrence of viral sequences within each batch: '1' denotes that at least one pool tested positive, and '0' denotes that all pools tested negative. Only viruses detected in at least one pool are listed. [3]The percentage of positive outbreak batches and the percentage of positive control batches are shown for each virus. [4]Outbreak and control batches were compared for the presence of viral sequences using Fisher's exact test with Holm-Šídák correction for multiple comparisons. [a]Columns in bold indicate non-diseased control batches.

Figure 3

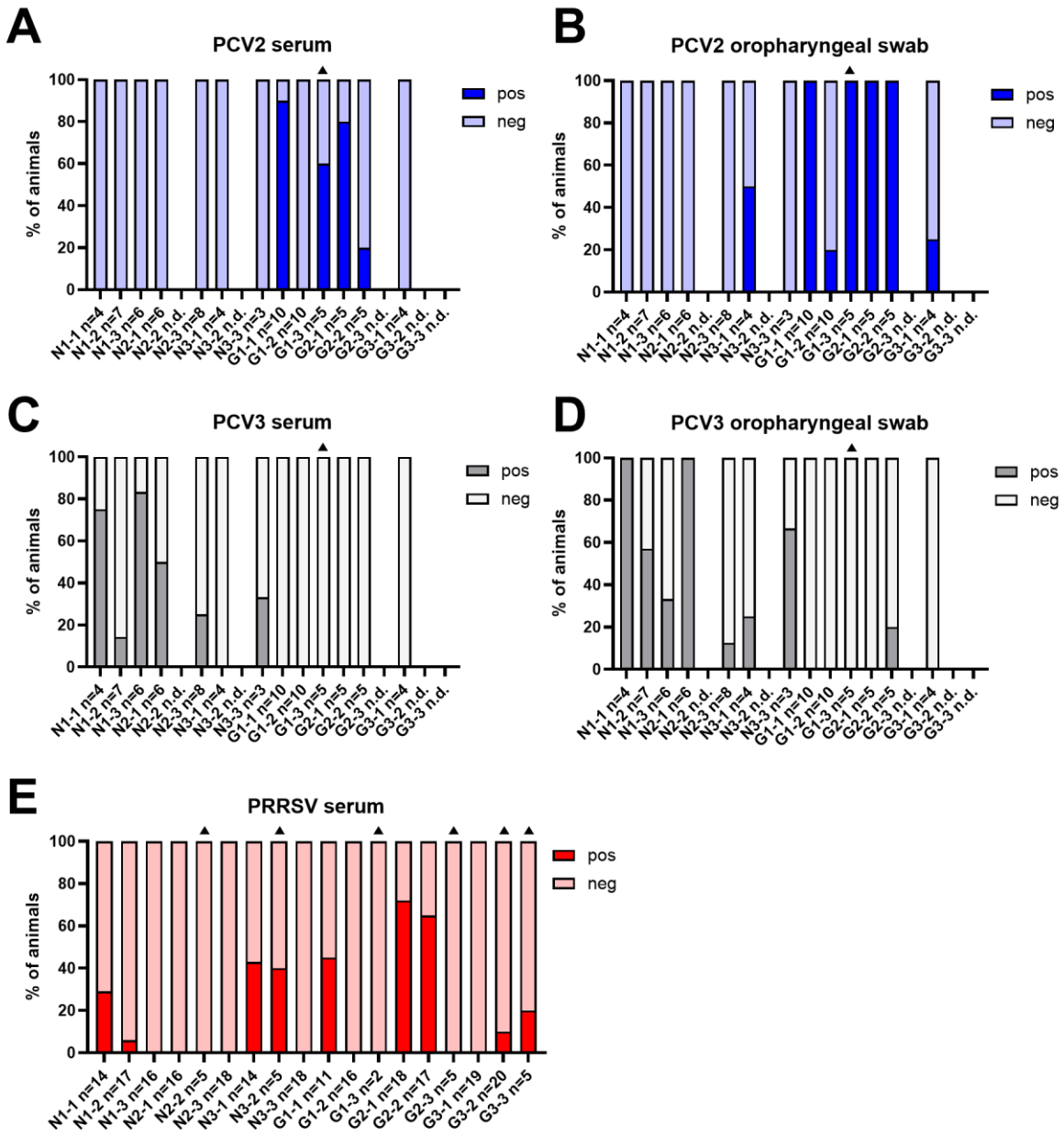


Figure 3: **Percentage of PVC2, PCV3 and PRRSV positive animals per batch as determined by qPCR.** A) and B) show the percentage of animals positive for PCV2 in respectively serum or oropharyngeal swab per batch. C) and D) show the percentage of animals positive for PCV3 in respectively serum or oropharyngeal swab per batch. E) depicts the percentage of positive animals for PRRSV in serum for each batch. The number of animals tested by qPCR is indicated in the bar label and control batches are marked with a triangle.

4. Discussion

The present study characterised consecutive *S. suis* disease outbreaks in nursery piglets on three German and three Dutch farms, with the aim of identifying risk factors for *S. suis* disease. Despite this targeted design, no consistent association was detected between S. suis disease outbreaks and either tonsillar DASS carriage or the concurrent presence of viral pathogens. These findings are discussed in the context of study limitations and the inherent complexity of *S. suis* disease epidemiology.

DASS was detected on all farms throughout the study, consistent with their selection based on a known history of *S. suis* disease, and the detected variable prevalence of DASS corroborates a previous report (5). At the batch level, a higher DASS/total *S. suis* ratio was observed in outbreak batches on two farms, but no consistent association between DASS carriage and disease was detected across farms. These findings are broadly consistent with those of Mugabi et al., who similarly found no association between DASS prevalence and *S. suis*-associated mortality, although their assessment was conducted in the preweaning rather than the postweaning phase (5). While DASS strains are associated with invasive disease (9), it remains unclear whether tonsillar carriage or higher oropharyngeal quantities of DASS increase the likelihood of a barrier breach and subsequent disease. Normalization of DASS quantities to total *S. suis* copies or total host-DNA in a swab sample may be necessary to accurately compare results between animals. Common pathogenic serotypes were cultured from invasive disease cases in the outbreaks, with the interesting observation that different serotypes were recovered from consecutive outbreaks on the same farm. Despite the introduction of an autogenous *S. suis* vaccine in sows on farm N1 before rounds 2 and 3, outbreaks still occurred in both production rounds. The applied autogenous vaccine only included serotype 2, while the consecutive outbreaks were dominated by serotype 9 for which likely no cross-protection would be established with this vaccine.

A total of 19 distinct viruses were identified, including PRRSV and PCV2 which have been suggested to predispose pigs to *S. suis* disease (8, 17). However, no significant association between the presence of viral nucleic acids and *S. suis* disease was detected by either metagenomic sequencing or specific qPCR. A critical contextual factor is that vaccination against PCV2 (piglets, 5/6 farms) and SIV (sows, 4/6 farms) was widely applied, and MLV PRRSV-1 sow vaccination was applied on all farms. Vaccination reduces viral circulation and may have attenuated the contribution of these viruses to *S. suis* disease risk in this study, potentially explaining the absence of a detectable association. Additionally, PRRSV-1-positive results should be interpreted with caution, as vaccine virus and field virus could not be differentiated. Noteworthy was the abundant detection of PCV3 in outbreak batches in The Netherlands (7/7). A statistically significant association could not be demonstrated owing to the lack of control batches analysed, but this finding may warrant further investigation. PCV3 was first described in 2015 and its pathogenesis remains poorly understood; clinical disease associated with PCV3 is thought to arise from a combination of co-infecting agents and environmental risk factors rather than from PCV3 infection alone (36, 37). Whether PCV3 predisposes the host to secondary infections or is itself a secondary finding remains unclear.

Several additional viruses were detected whose clinical relevance in this context is uncertain. Porcine endogenous retrovirus and porcine bocavirus were detected at high rates, consistent with previous reports (34). PRV-1, which has been recently detected to circulate in pigs with respiratory signs in Germany (38), was also detected here in three batches on three different farms. BVDV, primarily a cattle pathogen, was detected in half of the batches even though the farms had no relationship to cattle. BVDV has been previously shown to infect pigs (39), and due to its close phylogenetic relationship to other pestiviruses and the risk of diagnostic misinterpretation this high prevalence may be noteworthy to the field. SARS-CoV-2 sequences were detected in samples from two German and two Dutch batches; given the sampling period (2021–2022) and the fact that SARS-CoV-2 does not replicate productively in pigs (40, 41), this finding might reflect human-derived sample contamination. All detected viruses might shape the microbiological communities in the

upper and lower respiratory tract, and some of the major pathogens have shown associations with disease (i.e. Porcine respiratory disease complex) (42). While the tonsillar microbiome has been earlier studied in the context of *S. suis* disease (11, 43), the impact of the oropharyngeal virome may also warrant further investigation.

Methodological limitations affected the sensitivity and interpretation of viral detection. Pooling of samples reduced analytical sensitivity, and the ViroCap sequence capture approach does not provide viral load quantification. SIV was not detected, which may be due to pooling or use of oropharyngeal swabs which may be a suboptimal sample type compared to tracheo-bronchial swabs, nasal swabs and oral fluids (38, 44). A potential mismatch between Virocap capture sequences and circulating SIV strains is another possibility – but this could not be verified as capture sequences are not disclosed by the manufacturer.

A notable finding was the frequent discordance between clinical and post-mortem diagnosis of *S. suis* disease, which occurred in both directions. In Germany, only 33% of clinically suspected cases were confirmed post-mortem, compared with 75% in the Netherlands. This difference may be related to the timing of farm visits: German farms were visited at 4–5 weeks of age, shortly after weaning, whereas Dutch farms were visited at 7–8 weeks, which may better coincide with the peak incidence of *S. suis* disease. At the batch level, one German batch (G3-2) that was clinically classified as an outbreak batch was designated false positive, as no trCA were found upon post-mortem examination. *S. suis* was not isolated from any internal organ in this batch, and the clinical signs observed by the veterinarian may have been caused by other bacterial pathogens with a similar clinical presentation, such as STEC, *Glaesserella parasuis* and *Mycoplasma hyorhinis* (45-49). Notably, unconfirmed cases and their pen mates (‘OTHER’ group) had elevated CRP concentrations, indicative of systemic inflammation. This is consistent with the veterinarian’s observation that animals in this pen were diseased.

In addition, our strict trCA definition may have led to an underestimation of confirmed cases. In experimental infections, *S. suis* cannot always be re-isolated from challenged animals, and histopathological lesions are sometimes absent (50, 51). The limited sensitivity of culture is also suggested by our own data: *S. suis* was isolated from only 60% of oropharyngeal swabs, whereas 98% were positive by qPCR. Conversely, 5 of the 41 clinically healthy animals (CAC) that were necropsied yielded positive *S. suis* cultures with accompanying histopathological lesions, all from pens with confirmed *S. suis* cases. These animals may have been necropsied at an early stage of infection, before the onset of clinical signs, or may have had subclinical disease. Together, these findings indicate that clinical observation alone is an imperfect indicator of *S. suis* disease, and that post-mortem confirmation is important for reliable case classification.

Limitations and future directions

Several study limitations may have contributed to the absence of clear risk factor associations. The number of farms and batches was limited, and the distribution of outbreak and control batches was unbalanced across farms. Discordance between clinical and post-mortem diagnosis further complicated case classification and overall, only a limited number of trCA were identified. The limited number of trCA limited the power to detect an association between DASS carriage and disease at the individual level. Regarding the viral co-infections, it is important to note that the presence of viral nucleic acids was only measured at the time of sampling when the *S. suis* outbreak occurred. Thus preceding viral (or bacterial) infections and their sustained immunological effects were not evaluated yet may have played a predisposing role (52). Environmental and management factors, which were also not included in the analysis, may additionally influence *S. suis* disease outcomes (8, 53). Future

studies with sufficient power to detect associations, will be necessary to better characterise risk factors for *S. suis* disease under field conditions.

Conclusions

In this study we characterised consequent *S. suis* disease outbreaks in three Dutch and German farms. Clinical diagnosis of *S. suis* disease by the visiting veterinarian could only be confirmed by pathological and bacteriological analysis in a proportion of clinically classified cases. DASS was detected on all six farms, with highly variable batch-level prevalences. No consistent association was detected between *S. suis* outbreak status and either tonsillar DASS carriage or the concurrent detection of viral nucleic acids. Study limitations should be considered when interpreting these findings. Our findings highlight the complexity of *S. suis* disease and the considerable challenges of field-based risk factor studies.

List of abbreviations

BVDV; bovine viral diarrhoea virus
CA; case animals showing clinical signs of *S. suis* disease
CAC; case-associated control animals, i.e. apparently healthy pen mates of CA
CNAC; case non-associated control animals, i.e. apparently healthy animals in the same department as the CA but not in the affected pen
CON; control animals sampled in a control batch (no outbreak)
CRP; C-reactive protein
DAM1; disease-associated marker 1; sequence within SSU_RS01130
DASS; Disease-associated *Streptococcus suis*
G1; Germany farm 1
G2; Germany farm 2
G3; Germany farm 3
Hp; haptoglobin
N1; Netherlands farm 1
N2; Netherlands farm 2
N3; Netherlands farm 3
PCV2; porcine circovirus type 2
PCV3; porcine circovirus type 3
PERV; porcine endogenous retrovirus
PHEV; porcine haemagglutinating encephalomyelitis virus
PoSCV; porcine stool-associated circular virus
PRCV; porcine respiratory coronavirus
PRRSV; porcine reproductive and respiratory syndrome virus
PRV1; porcine respirovirus 1
SIV; swine influenza virus
TGEV; transmissible gastroenteritis virus
trCA; true case animals based on post-mortem examination (bacteriology & pathology)
trCAC; true case-associated control animals, i.e. apparently healthy pen mates of trCA
trCNAC; true case non-associated control animals, i.e. apparently healthy animals in the same department as the trCA but not in the affected pen
trCON; true control animals sampled in a true control batch (no outbreak)
TTSuV; Torque teno sus virus
ViroCap; targeted viral sequence capture
VM1; virulence marker 1; sequence within SSU_RS01130; corresponds with DAM1

Declarations

Ethics approval and consent to participate

Pig husbandry on all farms complied with Dutch and German husbandry regulations. All procedures involving animals were conducted in accordance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (European Treaty Series, No. 123). Animal owners were informed of applicable animal welfare regulations prior to and during the study period and provided signed data policy and protection declarations. The study was approved by the relevant national authorities: in Germany, by the Lower Saxony State Office for Consumer Protection and Food Safety (approval number: 33.8-42502-05-20A558); and in the Netherlands, by the Central Authority for Scientific Procedures on Animals and the local Animal Welfare Body of Wageningen University & Research (approval number: AVD 4010020198504/2018.D-0041). The guidelines of FELASA and ARRIVE were followed throughout. All sampling, treatment, and euthanasia procedures were performed by an experienced and qualified veterinarian, and all measures were taken to minimise unnecessary suffering at every stage.

Consent for publication

Not applicable

Availability of data and materials

All data supporting the findings of this study are included in this published article and supplementary table 1. Additional datasets are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors’ contributions

Conceptualization, P.H., N.S.-Z., J.R., L.D.P., P.V.-W., J.W., I.H.-P., M.V.; methodology P.H., N.S.-Z., J.R., L.D.-P., J.C., S.T., L.R., H.F., E.R, M.H., F.H.; formal analysis, P.H., N.S.-Z., J.R., J.C., E.R., M.H., J.W., I.H.-P., M.V.; investigation, P.H., N.S.-Z., J.R., M.H., E.R., L.W., J.W., I.H.-P., M.V.; data curation P.H., N.S.-Z., J.R., I.H.-P., M.V. writing—original draft preparation, P.H., I.H.-P. and M.V.; writing—review and editing, P.H., N.S.-Z., J.R., L.R., H.F., L.D.-P., S.T., L.W., J.W., I.H.-P., M.V.; visualization P.H., M.V.; supervision N.S.-Z., I.H.-P., M.V.; project administration, M.V., P.V.-W., J.W., L.W.; funding acquisition, N.S.-Z., J.W., P.V.-W., L.W.. All authors have read and agreed to the published version of the manuscript.

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Supplemental Figures

Supplemental Figure 1

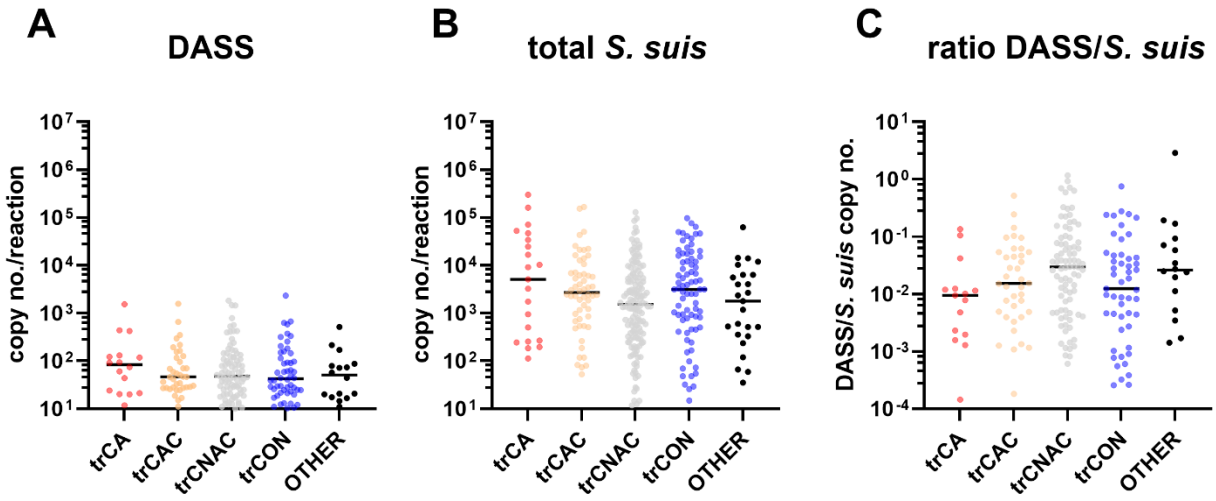


Fig S1: **Carriage of DASS and total *S. suis* in the oropharynx of the different classes of animals**.

A) Genome copy numbers of DASS in DASS-positive trCA (n=16), trCAC (n=38), trCNAC (n=87), trCON (n=52) and OTHER (n=16) animals. B) Genome copy numbers of *S. suis* in *S. suis*-positive animals from the different groups. Copy numbers depicted were corrected for the abundance of 16S rRNA target (4 copies per *S. suis* genome). C) Ration between DASS and total *S. suis* copy numbers in positive animals from the different groups. Genome copies of all groups were compared using a non-parametric Kruskal-Wallis test with Dunn's multiple comparisons test. Significant differences are indicated by asterisks, with * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.

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