# When to use next-generation sequencing for clinical and epidemiological decisions related to porcine reproductive and respiratory syndrome virus

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Next-generation sequencing (NGS) is a high-throughput sequencing technology that allows sequencing large amounts of DNA or RNA molecules. Like a polymerase chain reaction (PCR)-based assay that is commonly used to detect DNA or RNA with detection levels reflected by cycle threshold (Ct) values, the NGS is a promising tool in its ability to 1) sequence and recover large genomes (eg, viruses and bacteria) and 2) discover novel or previously unrecognized agents. Using massive parallel sequencing methods, NGS enables simultaneous sequencing of agents or strains in a sample. Several NGS technologies or platforms are available, eg, Illumina, Minion, Nanospore, etc, each with its unique sequencing approach, and descriptions of these technologies are out of the scope of this factsheet. Recent research advancements have significantly improved NGS turnaround to less than a day[1]; unfortunately, such technology is still commercially unavailable.

Use of NGS for porcine reproductive and respiratory syndrome virus (PRRSV) epidemiological characterization has become more popular in North America.[2-7] The ability to sequence the whole genome and multiple agents at a time differentiates NGS from prior technologies like the Sanger technique[8] or its modification.[9] Sanger technology has been largely used for PRRSV open reading frame 5 (ORF5) sequencing. The ORF5 has high genetic diversity compared to other more conserved regions of the genome and codes a glycoprotein involved in inducing neutralizing antibodies.[10-12] The ORF5 sequencing is commonly used by field veterinarians and decision-makers to determine genetic relatedness and diversity of PRRSV strains; PRRSV-2 ORF5 sequences have been classified using restriction fragment length polymorphism (RFLP) patterns and genetic lineages.[13-17] However, the ORF5 sequence only represents 4% of the PRRSV genome[18] and sequencing the whole genome via NGS can provide a more detailed genotypic characterization of a PRRSV genome. Like any other diagnostic test, NGS can succeed and recover a whole genome or fail and recover partial genomes or genome fragments named contigs. When a farm referent strain is present, contigs can still be very useful if appropriately analyzed to provide epidemiological information[6] and historical context of the PRRSV circulating in a population.

The success of NGS is dependent on the preparation and assembling method of the NGS library along with a myriad of additional factors:

### Sample type

Individual-based sample types, like serum and lung samples, are more prone to whole-PRRSV genome recovery. Population-based samples, eg, processing fluid and oral fluid samples, are more likely to recover genome fragments. However, generated sequences represent a sequence of nucleotides most prevalent at each position, which might not represent an actual virus present in the sample. Moreover, it might represent the most prevalent virus in the sample, which might differ from the most prevalent virus in the population if Ct differences are high enough. By employing parallel sequencing methods, NGS is more likely to detect more than one virus present in the sample.

### Viral load in the sample

Samples with a higher viral load, ie, the lower the Ct value the higher the viral load, are more prone to recover a whole genome.[6,9]

### Presence of single or multiple PRRSV genomes in a sample

If both are present in a sample, the NGS technique can successfully recover and distinguish PRRSV-1 and PRRSV-2 sequences[9,19]; however, when two or more similar PRRSV genomes from the same species (PRRSV-1 or PRRSV-2) are present in a sample, the recovery of a whole genome for each virus strain usually fails,[9,19] and contigs are usually recovered.[1,6] When two genomes of the same species are present in a sample, they may have a high degree of genetic similarity or different proportions or abundances in the sample, making it difficult to distinguish them from each other or from the background NGS noise.

Attempting virus isolation (VI) before submitting samples to NGS is an alternative for increasing the odds of obtaining a whole genome.[20,21] The downside of VI is that it could favor the selection of one viral strain if more than one is present in a sample and the isolated virus can accumulate genetic mutations during the replication required for the VI. Also, VI limits the ability of NGS

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to detect and sequence multiple PRRSVs if those strains are not captured during isolation or if one strain is favored during the isolation process. When more than one strain is present in a sample, performing VI and subsequent NGS on the isolate provides a method to thoroughly characterize the isolated strain. Furthermore, performing NGS in the original sample and comparing NGS-reported outputs with the isolate sequence can help further characterize multiple strains if present in the sample.

## Factors affecting NGS and examples of its use

Recovered whole PRRSV genomes can be used to compare PRRSV genome sequences, investigate recombination events, and identify the presence of substitutions, insertions, or deletions. However, most approaches described in the literature are not well suited for on-farm uses and interpretation of the results by veterinarians can be a challenge[2] due to the lack of user-friendly tools, the complexity of the data, and the required background knowledge. Additionally, the main limitation of whole-genome sequence interpretation for molecular epidemiology investigation is the lack of representative datasets, as whole-genome sequencing is not performed routinely and tends to be requested in a research-basis scenario.

Examples of potential farm and production system-level approaches[6] that may provide relevant insights into PRRSV genetic epidemiological characteristics using the NGS technique and bioinformatics analysis are described here.

When an outbreak occurs, individual samples, eg, serum or lung tissue, should be sent for traditional real-time reverse transcriptase-PCR (RT-PCR) to establish if the farm is PRRSV positive and to obtain the Ct values of the samples. The sample with the lowest Ct value, preferably one with a Ct value in the low 20`s or less, should be sent for NGS. The expected output is a whole genome that can be used as the farm-referent outbreak virus. As additional sequences are generated during routine monitoring and surveillance, this referent genome can be used for subsequent within- and between-farm PRRSV genome comparisons. If funding availability is an issue, banking the RT-PCR PRRSV-positive samples in long-term storage for future use may be an option. Whole genomes can be used to compile farm or production system databases from outbreaks over time, and comparisons can be made to identify the presence of mutations, genetic evolution, and detection of recombination events within the system. Investigation of mutations, genetic evolution, and recombination relying only on **contigs is not recommended.**

As the herd progresses toward stability, and unexpected RT-PCR PRRSV-positive results occur suggesting unexpected patterns of virus replication, NGS on RT-PCR PRRSV-positive samples can be used to investigate additional epidemiological information. Comparing NGS data from the current sample to the farm-specific outbreak virus allows for better insights such as:

- Did the virus evolve?
- Is the whole PRRSV genome a vaccine-like virus?
- Was there a recombination event?
- Was there an unrelated virus introduction?
- Are there levels of similarity with known highly virulent PRRSV strains, eg, Lineage 1A RFLP 1-7-4, Lineage 1C variant RFLP 1-4-4 (currently classified as L1C.5)?[17]

When a farm referent strain is present, the subsequent recovery of contigs is still useful for answering questions such as:

- Is there a vaccine virus vs a wild-type virus circulating in the herd?
- Is there evidence for co-circulation of multiple wild-type and modified-live virus (MLV) vaccine strains in the herd?

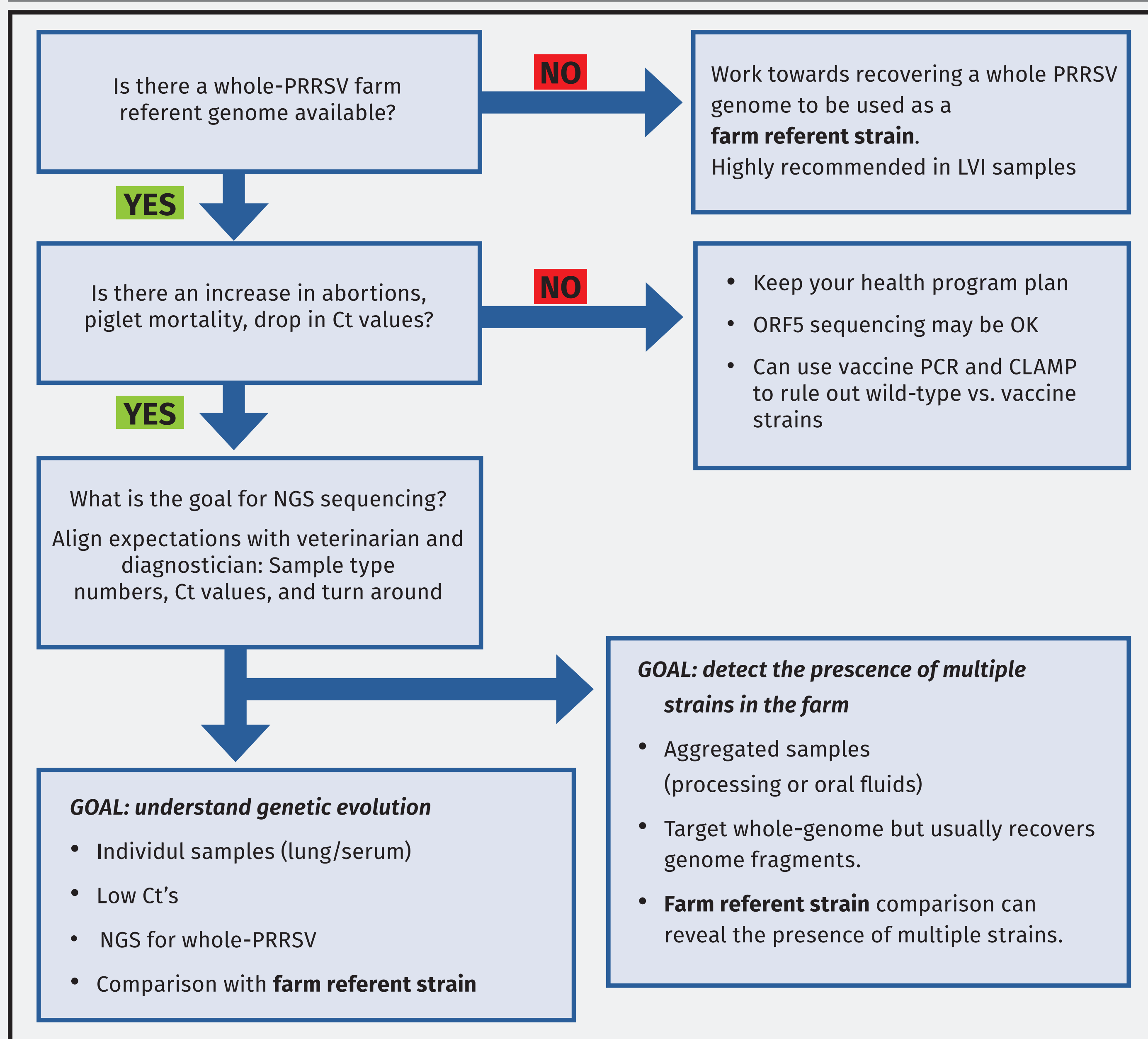
Next-generation sequencing can be used to differentiate vaccine and wild-type PRRSV. Outputs of NGS seek to identify underlying wild-type strains or recombination events outside the ORF5 region. Even though PRRS MLV vaccine-like PCR techniques and CLAMP[22,23] are alternative molecular techniques to differentiate wild-type vs vaccine strains, they target the nsp2 genome region or focus only on the ORF5 and cannot provide information regarding genetic changes that may have occurred in the remaining genome regions. The CLAMP is used to block the amplification of vaccine strains and increase the likelihood of amplification of other, non-blocked sequences that may be present.[23] Nevertheless, a US commercially available PRRSV MLV vaccine (PRRSGard, Pharmagate Animal Health) is a chimeric product derived from two distinct strains containing a 23-nucleotide insertion at the terminal region of the ORF1. The effective distinction between PRRSGard and wild-type strains requires a whole-genome sequence or a specific Sanger sequencing of the insertion region.

Another use case for NGS is the differentiation between vaccine and wild-type strains for downstream-placed pigs derived from breeding herds undergoing stabilization but with the unexpected appearance of clinical signs. Comparison with the referent strain from the breeding herd farm allows for an understanding of genetic evolution, detection of the presence of multiple strains, or both. For the case of multiple strain detection, further investigation may be required as this could be due to either additional strains from the breeding herd ("leaking" to downstream flows) or to lateral introductions into the growing flow.

The epidemiological question would play a role in designing future sampling and testing approaches. Appropriate sample collection and handling can contribute to better answers to the question of interest. If the interest is to understand genetic evolution, such as mutation or recombination events, then collecting, testing individual samples by RT-PCR, and conducting NGS on samples with low Ct values are more likely to result in the recovery of a whole PRRSV genome. If the interest is to detect the presence of multiple PRRSV genomes circulating in the herd, then using aggregated sample types, eg, oral or processing fluid, for NGS is more likely to recover contigs. When compared with a farm-referent strain, contigs can identify additional PRRSV genomes if present in the sample. Unfortunately, contigs are not well suited for genetic evolution analysis (Figure 1).

In conclusion, the growing use of NGS and the perceived importance of generated outputs for PRRSV epidemiological investigations is a reality. Constructing farm or system-based PRRSV whole-genome libraries will open the next frontier in understanding PRRSV epidemiological characteristics and evolution. Information generated through NGS can be used in the decision-making process of PRRSV control and intervention strategies.

**Figure 1:** One potential approach on how to use NGS

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