Porcine reproductive and respiratory syndrome (PRRS) diagnostics: Interpretation and limitations

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Summary

Porcine reproductive and respiratory syndrome virus (PRRSV) has been a major pathogen associated with swine disease. Attempts to control and better understand this disease have led to the extensive implementation of diagnostic assays. Techniques commonly used include enzyme-linked immunosorbent assay, indirect fluorescent antibody, serum neutralization, polymerase chain reaction, and nucleotide sequencing. These assays provide information about the PRRSV status of an animal or herd, but if not interpreted correctly, erroneous conclusions may be drawn and inappropriate decisions made in attempts to control PRRSV. Factors such as laboratory variation, reagents used in the assay, protocol variation, and technical limitations should all be considered when interpreting diagnostic results. The diagnostic assays based on nucleotide sequence also appear to be greatly misunderstood in the industry and do not provide insight into either PRRSV isolate virulence or vaccine selection. This manuscript attempts to review general diagnostic assay principles, provide insight into assay limitations, and provide recommendations on proper use of assays and interpretation of results.

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This article is the result of review and discussion of porcine reproductive and respiratory syndrome (PRRS) diagnostic techniques and technology by a panel of contributors. These individuals are members of swine diagnostic laboratories, scientists, or both, who have been involved with PRRS detection and monitoring. The goal of the panel was to define the uses and limitations of the technology, attempt to standardize reporting of diagnostic assays when possible, and, on the basis of the technical limitations of the assays, recommend proper uses of diagnostic assay results.

Since it was first reported in the early 1990's, porcine reproductive and respiratory syndrome virus (PRRSV) has become a pathogen of major economic significance in the global swine industry. Control of PRRSV requires the use of diagnostic assays to develop management strategies. The tools available to swine veterinarians for PRRSV detection have changed considerably. Initially, routine diagnostic testing was limited to virus isolation and serological assays (serum virus neutralization (SVN) and indirect fluorescent antibody (IFA) tests). Over the past 5 years, significant technical advances have not only led to improved PRRSV detection systems, but have also made these technologies commonly available to veterinary diagnostic laboratories and swine veterinarians. The new technologies include enzyme-linked immunosorbent assay (ELISA), reverse transcriptase polymerase chain reaction (RT-PCR)-based assays, restriction fragment length polymorphism (RFLP), and sequence analysis (sequencing and dendrograms). The complexity of PRRS management has also increased over time as swine practitioners attempt to maintain PRRSV-negative herds, control acute PRRS outbreaks, differentiate between modified-live-virus (MLV) vaccines and field virus, and stabilize positive herds.

It is critical that swine veterinarians understand the technology associated with the diagnostic assays currently available for PRRS. Proper use of the assays can provide invaluable information for PRRS control. Improper use or "over-interpretation" can generate poor decisions resulting in failure of PRRS control efforts. The goal of this review is to provide the swine veterinarian with an overview of the key technologies for PRRS and a summary of their uses and limitations.

PRRSV serological assays

Assays for detecting serum antibodies against PRRSV include the ELISA, IFA, and SVN tests, and the immunoperoxidase monolayer assay (IPMA).[1] The IPMA is not used routinely in diagnostic laboratories in North America and will not be discussed in detail.

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PRRS ELISA

The commercial PRRS ELISA (HerdChek; IDEXX Laboratories Inc, Westbrook, Maine) uses an indirect format to detect serum antibodies against certain PRRSV antigens. Per kit instructions, a sample-to-positive (S:P) ratio ≥ 0.4 is considered positive (indicates presence of antibody to PRRSV) and has reported sensitivity and specificity of 100% and 99.5%, respectively.[2] The kit contains both North American and European PRRSV-antigen types, although the exact nature of the antigen is proprietary.

The HerdChek ELISA has a number of advantages over some other assays. First, it is licensed by the United States Department of Agriculture and the Canadian Food Inspection Agency. Other advantages include uniformity in manufacturing, rapid results, and the ability to identify antibodies against both European and North American PRRSV strains. Extensive information on kit components, basis for cut-off values, calculations, and kit recommendations are available from the vendor. Because of the assay's standardized format, it is very reproducible.

In the laboratory, some problems have been noted. Correct preparation of the initial serum dilution requires a high level of precision on the part of the technician, and quality control (QC) in the manufacturer's ELISA software is sparse (ie, software may fail to identify QC problems).

The HerdChek PRRS ELISA is the most commonly used serologic assay for antibodies against PRRSV, and most practitioners are familiar with its use and interpretation. Interpretation of the results is relatively straightforward. If the S:P ratio is <0.4, the sample is classified as negative for PRRSV antibodies. If the S:P ratio is ≥0.4, the sample is classified as positive. Over-interpretation, such as failure to appreciate the degree of variation in the immune response among various animals, and over-interpretation of the significance of differences between S:P ratios, can lead to erroneous conclusions at both the animal and herd level.

Other ELISA tests

Other ELISA formats have been described, and at least two other ELISA kits have reached the commercial market: an indirect ELISA (CIVTEST Suis PRRS; Laboratorios HIPRA, SA, Girona, Spain), and a blocking ELISA (Bio-Vet PRRS-Blocking; Bio-Vet Laboratories, Saint-Hyacinthe, Quebec).

IFA test

The IFA test is performed by placing the serum sample on virus-infected cells in the wells of microtiter plates, then looking for evidence of an antigen-antibody reaction by the subsequent addition of fluorescein-labeled anti-porcine antibodies. The IFA has good specificity, but sensitivity of the assay is dependent upon several factors, including laboratory variation. Factors that may vary in the laboratory include media, protocols, incubation times, technician skill, cell types used in the assay, the subjective interpretation of the reaction by the technician reading the results, and the degree to which the PRRSV isolate used in the assay differs antigenically from the isolate infecting the pig, especially in herds which may be infected with European serotype.

An advantage of the IFA over the ELISA is that the magnitude of the antibody titer can be estimated. Particularly if the homologous virus isolate infecting the pig is used in the IFA, the assay can reliably detect specific antibodies for approximately 3 months post-infection.[1]

SVN test

The SVN test is also a cell-culture-based assay in which a known level of virus is incubated with various dilutions of test serum. After incubation, serum is placed on a sensitive cell line to determine the titer of the antibody which was able to neutralize the PRRSV. There is no standardized protocol or set of reagents used with this assay, and most laboratories have developed similar but independent systems. It is less sensitive than IFA or ELISA because neutralizing antibodies against PRRSV develop slowly post-infection (>21 days). In addition, some pigs develop relatively low neutralizing antibody titers.[3] The interpretation and application of SVN results to field situations is problematic, partly because the correlation between neutralizing antibody and immunity or protection has not been demonstrated. The general assumption often is made that the presence of neutralizing antibodies means that virus is no longer being shed. This may be true in some cases, but it is also true that some PRRSV-carrier animals have high levels of neutralizing antibodies.

The SVN test is more expensive and more time-consuming than other PRRS serological assays, and is technically difficult to perform. Few laboratories perform this test on a routine basis. Results of SVN tests should be interpreted with caution, because the PRRSV isolate used for testing may affect results and cause confusion when data from various laboratories is compared. The magnitude of the serum neutralizing response may be higher when the infecting isolate is used in the neutralization assay than when a different isolate (heterologous) is used. Although there is a high degree of homology among most North American (non-European) isolates, subtle differences may affect the magnitude of SVN and IFA results. In general, the SVN assay should be considered a research tool.

Western immunoblotting

Western immunoblotting is a research tool that has sporadically been used to further investigate suspected false-positive serologic results. Native or recombinant PRRSV proteins are separated by electrophoresis, then the proteins are transferred to nylon or nitrocellulose membranes. The membranes are then incubated with the test and control sera. Antibodies binding to specific proteins are visualized using conjugated enzymes and appropriate substrates. The appearance of specific bands that correlate with known viral proteins indicates a positive test. The immunoblotting assay requires great care and numerous controls to avoid false-positive or false-negative reactions. The assay is also quite expensive and time consuming, thus limiting its application in routine diagnostic testing, and it should not be considered for routine use by swine practitioners.[4]

Interpreting results of serology assays

Several limitations should be taken into account when interpreting PRRS serology:

- Serological information from a single serum sample is not sufficient for diagnosing clinical PRRS in an individual animal because PRRSV infection is so common. For the same reason, positive results may or may not mean that PRRSV caused clinical disease. Current serologic assays can be used to measure PRRS exposure and MLV vaccine compliance (ie, to confirm that the pigs were vaccinated),

but cannot differentiate vaccine-derived antibodies from field-isolate-derived antibodies.
- Broad antigenic variation among PRRSV isolates is a concern in some tests. In particular, IFA and SVN test results are greatly influenced by the degree of antigenic relatedness between the isolate of virus used in the test at a diagnostic laboratory and the isolate infecting the pig.
- At the present time, it is not possible to accurately predict the shedding or carrier state of an individual animal on the basis of serologic assays.
- Finally, failure to appreciate the degree of variation in the immune response among various animals can lead to erroneous conclusions or "over-interpretation" of HerdChek ELISA S:P values.

Testing for PRRSV by RT-PCR and RFLP

RT-PCR testing

The RT-PCR is used to detect the genetic material (genome) of PRRSV in swine tissue homogenates and in clinical specimens, including semen, serum, oropharyngeal scrapings, and lung lavage samples. Viral RNA is detected through extraction of RNA from the sample, a reverse transcriptase (RT) step converts the RNA to DNA, and DNA is exponentially amplified by PCR. The RT-PCR can be accomplished within 1 to 3 days after receiving a diagnostic specimen for testing. The most conserved genes are targeted for detection of PRRSV by RT-PCR. The open reading frame (ORF) 6 and ORF 7 areas of the genome are the most conserved and therefore the most common genes detected. However, laboratories may use one or more different target genes, such as ORF 5, in sequencing and determining the RFLP cut pattern.

The advantages of using the RT-PCR procedure are rapid turnaround time and high sensitivity and specificity. A disadvantage of RT-PCR is that the viral genome might not be detected if there are significant genetic differences between the PRRSV and the primers being used in the assay. For example, some PCR primers detect US PRRSV strains, but not European or European-like strains. Good technical skills and laboratory procedures are also essential to obtain reliable interpretation of PCR test results.

The RT-PCR methods have changed in recent years and include automated, one-tube fluorogenic systems, such as "TaqMan" (Perkin-Elmer Applied Biosystems, Inc, Foster City, California), as well as the "molecular beacon" RT-PCR technique. The automated RT-PCR uses fluorescent probes that bind to the RT-PCR product as it is being made (ie, "real-time" RT-PCR) and are used in a 96-well plate format on an instrument called a Perkin-Elmer ABI PRISM 7700 Sequence Detection System. New advances in instrumentation will likely continue and practical applications of real-time PCR may soon be available from some diagnostic laboratories.

As with any PCR testing, it is important to determine the sensitivity and specificity of the assay, and to validate the system on the basis of experimental PRRSV infections as well as field samples. Several critical factors may affect the sensitivity and specificity of RT-PCR testing. These factors include, but are not limited to, technician training or skill level, type of specimen (semen, sera, tissues, cell culture), specimen condition, specimen volume (eg, 100 μL versus 10 mL), specimen processing, extraction technique, primers, and cycle optimization. This information, and in addition a description of controls and standards routinely used to ensure assay quality, should be readily available from the diagnostic laboratory to which the samples are submitted.

In PRRSV test and removal programs, RT-PCR has been used to detect viremic animals or carriers, primarily using serum, tonsil biopsy, or oropharyngeal scrapings. This assay has also been used to detect PRRSV RNA in semen samples, and has an advantage over the virus isolation technique because of the toxicity of semen on the cells used in the virus isolation assay. Because RT-PCR detects a portion of the genome of an infectious agent, it is not necessary to wait for a host immune response before the virus can be detected. Therefore, in the case of acute infections, utilizing RT-PCR allows the PRRSV to be detected earlier than if a serological test were used. For comparisons of PRRSV field isolates and vaccines, an RT-PCR assay targeting ORF 5 is also used to obtain nucleotide sequence information and RFLP patterns.

Interpreting RT-PCR results

- Theoretically, RT-PCR tests are highly specific and sensitive.[5] However, test performance within and between laboratories may be affected by several factors, such as laboratory personnel training and experience, and type and condition of specimens, as well as differences in procedures, including extraction and primer design.
- Positive RT-PCR results may not necessarily indicate the presence of replicating virus in the sample. However, there is a high correlation between RT-PCR results and detection of replicating virus, indicating the increased likelihood that the assay is detecting actively replicating virus.[6]
- Positive ORF 5 RT-PCR samples may readily be used for subsequent evaluation by RFLP and sequencing.
- The RT-PCR technique can be used effectively, in combination with serology, to screen and monitor PRRSV-negative pig herds, and to screen breeding stock before movement.

RFLP analysis

Analysis of PRRSV by RFLP is performed on the RT-PCR product after digestion of ORF 5 using three restriction enzymes. A three-digit code based on cutting patterns may then be assigned to the isolate (eg, 1-4-2). This technique was originally designed for use in PRRSV research trials to differentiate vaccine strain (Ingelvac PRRS; Boehringer Ingelheim, St Joseph, Missouri) from challenge virus.[7] Although the technique cannot differentiate vaccine virus from its parent, it has been applied in the field to help differentiate Ingelvac PRRS vaccine strain (RFLP pattern 2-5-2) from various field isolates. The RFLP test is a valuable tool that can be rapidly performed on PRRSV isolates or samples (serum or tissue) at relatively low expense. This information may then be used to track introduction of a new isolate into a swine system, monitor spread, and in some cases, differentiate vaccine (Ingelvac PRRS) and field virus by differences in their RFLP cut patterns.

Over the past few years, another MLV vaccine (Ingelvac PRRSV ATP; Boehringer Ingelheim) has become available, with an RFLP pattern (1-4-2) that cannot be differentiated from common field virus

patterns. It has also been determined that MLV vaccines may change over time, especially after in vivo passage.[7] As a result, "gray" (inconclusive) RFLP patterns have been identified (for example, 2-1-2, 2-6-2, and 1-5-2) that result in inconclusive interpretation of the RFLP test result unless there is supportive information on herd histories and subsequent diagnostic monitoring. Isolates yielding "gray" RFLP patterns are sometimes sequenced in an attempt to provide additional information.

The RFLP cut patterns currently in use are based on three restriction enzyme cut sites of the ORF 5 region: their significance in pathogenesis is unknown. The ORF 5 region represents a very small portion of the total genome, and change in a single base pair can alter the RFLP cut pattern. In some cases, an understanding of the "RFLP key", and access to it, may be necessary to assess the significance of a change in RFLP patterns.[7] Changes that may occur include a change in a single base pair, changes in multiple base pairs, or loss of a cut site. For these reasons, it is clear that RFLP should not be used to assess relative virulence of PRRSV strains or selection of a vaccine.

Interpreting RFLP results

- The RFLP on RT-PCR products of PRRSV may be used to screen large numbers of samples to evaluate PRRSV isolates and spread of isolates in a herd, or introduction of PRRSV.
- In some cases, PRRSV RFLP may be used in vaccinated herds to differentiate vaccine isolates from field isolates, depending on the vaccine(s) used and field virus patterns within the herd. It must be noted that PRRSV cut patterns may change during in vivo replication.
- Results of PRRSV RFLP provide no indication of relative isolate virulence (eg, PRRSV RFLP 1-4-2 does not absolutely correlate to a "hot" strain).
- As the RFLP numerical system does not correlate with vaccine protection or efficacy, PRRSV RFLP profiles of field samples should not be used to make a vaccine selection.
- Changes in RFLP pattern should be interpreted with care. Changes may include single or multiple base pairs or addition or loss of a site.
- Caution must be used in comparing RFLP patterns, as ORF 5 comprises only 4% of the PRRSV genome, and therefore identical RFLP patterns in no way conclusively demonstrate relatedness over the remainder of the genome. This test is most useful for investigations within a herd or system.
- Inconclusive ("gray") RFLP patterns continue to emerge. As many as 13 different cut patterns have been seen with one enzyme.
- Nucleotide additions and deletions would not be noted with RFLP.

Virus sequence analysis

Sequencing

Sequencing of PRRSV produces an exact nucleotide sequence from the ORF under analysis. Commonly, the diagnostic laboratory provides veterinarians and researchers with a report of the sequence aligned to commercially available MLV vaccine strains. The laboratory also predicts the RFLP pattern that would be generated with restriction enzymes designed by Wesley et al.[7] Sequence analysis provides investigators with the exact sequence of part of the submitted isolate, which may show nucleotide mutations, additions, and deletions missed by RFLP analysis. Sequencing also provides a tool for veterinarians monitoring the status of large herds over time. The diagnostic laboratory may produce farm-specific dendrograms on request.

The usefulness of sequence analysis in differentiating PRRSV isolates was initially demonstrated by Kapur et al[8] when they conducted a study to describe PRRSV strain variation. From their analysis of ORFs 2 to 7, they were able to predict that ORF 5 was the most variable gene and ORF 6 was the most conserved gene. This is the basis for the selection of the ORF 5 and 6 target gene(s) for sequencing. The investigators also predicted that the substantial differences in nucleotide diversity among all of the ORFs suggested that the virus was evolving by processes other than simple accumulation of random neutral mutations. They provided statistical support for the hypothesis that intragenic recombination or gene conversion takes place in ORFs 2, 3, 4, 5, and 7, but probably not in ORF 6. Intragenic recombination has now been shown to occur in vitro[9] and in vivo.[10] Rates of change are relatively similar in both the structural (ORFs 2 to 7) and non-structural regions (ORF 1a and 1b). Although sequencing can be performed on any region of the genome, from a diagnostic point of view, ORF 5 is preferred because of the extensive data bank on this region, the ability to monitor change over time, and the fact that ORF 5 is highly variable (extremely polymorphic). Isolates with a 2-5-2 RFLP pattern have been evaluated by sequence analysis. Sequence results suggest that some of these isolates are not vaccine or VR 2332 (parental strain) derivatives, but in fact represent wild type strains of circulating PRRSV (M.P. Murtaugh and K.S. Faaberg, unpublished data, 2000).

Modified live PRRSV vaccines have now been available for several years, and PRRSV has continued to evolve and spread. It has become apparent that sequence analysis of a short region (ORFs 5 to 6) of an isolate cannot provide concrete evidence of MLV-vaccine reversion in the field. Modified live vaccines have been shown to spread, co-exist within animals with other PRRSV strains, and undergo evolutionary and recombinatory pressures. Porcine reproductive and respiratory syndrome virus sequence changes within 1% or less are consistent with known amounts of change over short periods of time (100 days to 2 years), as defined by experimental studies.[11–13] Rates of change outside this range would be consistent with the isolate having no direct relationship to the reference virus used in the consensus sequence (eg, vaccine, VR 2332).[14] The rates of change are relatively consistent between the structural and non-structural regions, though information on the non-structural regions is limited.[14]

Selection of the sample for sequencing may also have an impact on the final result. Use of a PRRSV isolate from cell culture may bias the sample toward isolation of vaccine virus (which is cell-culture adapted) rather than isolation of a field isolate, particularly immediately after vaccination. Direct sequencing of positive RT-PCR products can be performed in most diagnostic laboratories and is the best approach, as it prevents isolate bias and mutations or change that may occur during the cell culture passage process.

As the genomic region(s) encoding PRRSV pathogenesis are not yet defined, and evolutionary pressures on the virus are poorly understood, diagnosticians should not provide definitive statements concerning the origin of a field isolate on the basis

of regional genomic sequence analysis alone. The best use of genetic sequence interpretation is to show relatedness of strains over time or within a herd on the same farm. This topic has been previously reviewed.[15]

Interpreting sequencing data

- Genetic sequencing is a valuable tool when used to monitor PRRSV strains within a farm or herd over time, and to show relatedness or monitor change. Sequencing can identify changes not identified by RFLP, for example, nucleotide insertions and deletions.
- Direct sequencing is the best option, as it provides sequence data not biased by cell culture selection of isolates or mutation that may occur in vitro. On the basis of experimental trials and noted levels of genetic change within PRRSV ORF 5, sequence changes greater than 1% suggest that two PRRSV isolates are not closely related. Genetic sequencing is probably the best tool available today to differentiate vaccine virus in herds with a complex PRRS history.
- The role of PRRSV genomic regions in pathogenesis are yet to be defined. The significance of changes reported is unknown and cannot be used to assess virulence or biological properties of the virus.
- Finally, sequence homology between field isolates and vaccines is not an accurate predictor of vaccine efficacy and should not be used to select a vaccine.

PRRSV phylogenetic (dendrogram) analysis

Phylogenetic (dendrogram) analysis is performed by aligning the nucleotide sequence or predicted amino acid sequence information of a set of PRRSV samples in an ordered fashion according to the similarity of pairs of sequences. Computer programs, notably Clustal V[16] and Clustal X,[17] compare all possible pairs of sequences and groups to identify the most similar pair; the program then compares this set to all remaining sequences. It continues this process until a tree is constructed (the dendrogram). The computer programs assume that all differences among sequences are due to random, independent mutations during evolution of the virus. Biologists have learned that sequence similarity corresponds to evolutionary history (phylogeny) of an organism. Therefore, dendrograms are commonly thought to represent or reflect ancestral relationships. In PRRSV, various regions of the genome, including ORFs 3, 5, 6, and 7, have been used to predict relationships in various studies.

This technique was originally adapted to PRRS research to predict how isolates were interrelated and to characterize the variation existing in PRRSV at the time of its discovery. The technique is exquisitely sensitive, since it can show differences between isolates as small as a single nucleotide. It may be used in the field to determine if the reappearance of PRRSV on a farm is due to the introduction of a new strain or to the reemergence of a strain that existed previously on the farm. It may be used to determine if PRRS outbreaks on a farm are due to a single clone of virus or more than one clone. In addition, it has been used to analyze supposed claims that vaccine has reverted to virulence. As with RFLP, this information can be used to track isolate introduction of PRRSV into a swine system, monitor spread, and in some cases, differentiate vaccine and field virus.

Phylogenetic (dendrogram) analysis is a powerful method for deducing evolutionary relationships when the history of a species is known or when the mechanism of evolution is known to be random mutation. However, nonrandom mutation, as exemplified by recombination, greatly skews phylogenetic software programs and can render dendrograms highly questionable. Similarly, genetic selection, perhaps by immunologic resistance, can effect changes that may not be genetically significant, but still show up as different branches on a phylogenetic tree.

Interpreting dendrogram analysis

- Dendrogram analysis is a powerful method for deducing evolutionary relationships between PRRS isolates when change is associated with random mutation.
- Dendrograms cannot be used under any circumstance to assess biological characteristics of PRRSV-like virulence. They can be used to show the possibility that an isolate is related to previously analyzed field isolates, and potentially to differentiate it from vaccine virus.
- Dendrograms are most effectively utilized at the farm or herd level to assess isolate introduction into a herd, monitor spread, monitor change over time, and in some cases, differentiate vaccine from field virus.
- Dendrograms are best used to evaluate isolates within a herd over time. If a single diagnostic case is to be evaluated, use of, and comparison to, a reference panel should be requested.
- Caution should be exercised in dendrogram interpretation. Consider the following events which may affect the interpretation of the data. Recombination and immunologic pressure are non-random events and might render the dendrogram inconclusive. Genomic regions of analysis (conserved or non-conserved regions) may affect the results of the dendrogram. Dendrograms do not indicate relative isolate virulence, and should not be used to select a vaccine strain.

Implications

- In this battery of tools for PRRS diagnosis, it is important to understand the benefits and limitations of each assay to insure proper interpretation.
- Serological tools, such as ELISA, provide excellent qualitative information on the PRRS status of a herd and provide a standardized format for testing. Care should be taken not to over interpret the S:P ratio beyond a herd positive or negative status.
- Veterinarians must have a good understanding of the technical limitations associated with PCR to insure valid use and application. Use PCR in combination with serology when monitoring negative herds and breeding stock.
- Nucleotide-sequence-based assays, such as direct nucleotide sequencing, RFLP, and dendrograms, do not provide an indication of isolate virulence or indications of vaccine selection.
- Nucleotide sequence data is most useful when used within a herd over time to monitor the changes that occur within a population or the introduction of new PRRSV isolates.
- Diagnostic tools are only as good as their valid and reliable use, and the

careful interpretation of their results.

- Veterinarians should work closely with the diagnostic laboratory and have a good understanding of the controls used, quality controls in place, and proper samples to be sent for testing.
- When evaluating the levels of genetic change between PRRSV ORF 5 isolates, identifying sequence changes greater than 1% suggests that the two PRRSV isolates are not closely related.

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